Proceedings
of the
Society
for
Experimental Biology and Medicine

Vol. 79 March, 1952 No. 3

SECTION MEETINGS

DISTRICT OF COLUMBIA	
George Washington University	February 7, 1952
CLEVELAND	
University Hospitals, Cleveland	February 14, 1952
ILLINOIS	
Michael Reese Hospital, Chicago	February 12, 1952
SOUTHERN CALIFORNIA	
University of Southern California	February 7, 1952

Aureomycin, Chloromycetin and Terramycin in Treatment of Acute Viral Hepatitis.* (19370)

James W. Colbert, Jr., Luitgard Bungards, and Marjorie Knowlton.
(Introduced by J. L. Melnick.)

From the Section of Preventive Medicine, Yale University School of Medicine, and the 98th General Hospital, European Command, U. S. Army of Occupation in Germany

The reports of the effectiveness of aureomycin, chloromycetin and terramycin against rickettsial and presumably viral diseases suggested a trial of these antibiotics in the management of acute viral hepatitis. The following is a report of the investigation of the use of these drugs in treatment of viral hepatitis at the Hepatitis Center, 98th General Hospital, U. S. Zone of Occupation, Germany.†

Materials and methods. Subjects. The patients studied in this investigation were male enlisted personnel of the United States Army of Occupation in Germany who were admitted to the 98th General Hospital within the first 10 days of viral hepatitis. It is quite probable that many of these cases were examples of homologous serum hepatitis but, in view of the fact that there are no means at present for differentiating infectious hepatitis from serum hepatitis clinically, the cases have all been classified as viral hepatitis. The diagnosis was made on the basis of characteristic symptoms and signs accompanied by consistent deflection of appropriate tests of hepatic function. All of the patients studied were jaundiced at the time of admission and the subsequent course of disease was mild or moderately severe. Twenty-four patients received 1.0 g of aureomycin orally every 6

---

* This represents work done under the auspices of the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

† The authors express acknowledgment to Lt. Col. Helmuth Sprinz, Director of Laboratories, 98th General Hospital, for assistance in this work, and to Dr. Richard J. Meyer and Dr. F. M. MacDonald for their participation.

hours for 10 days.‡ Four additional patients received the same dosage orally and 100 mg of aureomycin intravenously every 6 hours for 10 days. Ten patients received orally 50 mg of chloromycetin per kilo of body weight initially and 500 mg every 6 hours for the subsequent 10 days. Terramycin was given orally to 20 patients—1.25 g every 6 hours for 10 days.§ Fifty-four control patients, chosen alternately at admission, received an appropriate placebo by mouth every 6 hours for 10 days. *Management of patients.* The patients were examined daily and observations concerning pertinent symptoms and signs of disease were made. The weight was measured each week. The following tests of hepatic function were made at admission to the hospital and twice a week throughout the period of hospitalization: serum bilirubin (1′ direct and total); cephalin- cholesterol flocculation; thymol turbidity test; γ globulin turbidity test. After the 1′ direct serum bilirubin measured 0.4 mg % the retention of intravenously administered bromsulphalein in the blood was measured each week until 2 determinations of 6.0% or less were obtained. Except for the administration of antibiotics, both treated and control groups were treated in the same way. The diet contained 4200 calories, consisting of 190 g protein, 607 g of carbohydrate and 114 g of fat, supplemented with vitamins and brewer's yeast. Intravenous infusions of glucose were given as required. All patients were kept in bed, with the privilege of going to the bathroom, until all symptoms and signs of hepatitis had disappeared and the retention of bromsulphalein in the blood measured 6.0% or less. They were then allowed to be ambulatory for one week and if there was no evidence of relapse, either clinically or as determined by tests of hepatic function, they were discharged from the hospital either to duty or to a convalescent center.

Results. Clinical observations. There was no significant difference in the incidence or duration of complaints or physical signs of disease in the patients who received antibiotics and the control subjects. Hepatomegaly, anorexia, nausea, hepatic tenderness were the most common findings. The absence of fever and the limitation of abdominal pain in the majority of patients to the first week of disease precluded the possibility of observing the effect of the antibiotics on these points.

Laboratory observations. In general, the patients who received aureomycin had a slightly longer duration of abnormal results in the various tests of hepatic function (Table I). Serum bilirubin, cephalin-cholesterol flocculation and retention of bromsulphalein became normal somewhat earlier in the untreated patients. The same was true of the chloromycetin treated group except for the retention of bromsulphalein which became normal a few days earlier in the patients receiving the antibiotic (Table II). The difference was, however, not significant. Laboratory tests of hepatic function in patients receiving terramycin returned to normal earlier than in the untreated group (Table III). The difference in the two groups is particularly noticed when comparing the time required for the serum bilirubin and bromsulphalein dye retention tests to become normal. In analyzing the mean differences between the treated and untreated patients in relation to these 2 tests the t value in both instances is found to be greater than 2.0. Comparison of the means for the serum bilirubin test indicates a t value of 2.2 and for the bromsulphalein test the value is 2.3. The probability therefore that these means differ by chance alone is less than 5%. In examining the records of these patients it appeared that 4 *terramycin control* patients exhibited a maximum serum bilirubin concentration above 15 mg % while among the terramycin treated patients only one patient demonstrated such a high level. If the serum bilirubin concentration indicates the severity of disease, then the control patients may have been more seriously afflicted. Therefore a reanalysis was done after excluding the values of the 5 patients exhibiting such a high bilirubin concentration. The mean differences between the two groups still remained significant at less than the 5%

---

‡ Aureomycin was generously supplied by the Lederle Co.

§ The terramycin was generously supplied by the Charles Pfizer Co. The assistance of Dr. Gladys Hobby of that Company is gratefully acknowledged.

TABLE I. Comparison of Course of Liver Function Tests in 24 Patients Treated with Oral Aureomycin and in 24 Control Patients.

	Aureomycin treated						Control group					
		Days after start of therapy when values became normal						Days after start of therapy when values became normal				
Liver function tests	A No. of pt	Mean $\bar{x}$	σ	$\sigma_{\bar{x}}$	B No. of pt	C No. of pt	A No. of pt	Mean $\bar{x}$	σ	$\sigma_{\bar{x}}$	B No. of pt	C No. of pt
Total serum bilirubin	24	29.1	23.1	4.7	0	0	24	24.2	17.3	3.5	0	0
Bromsulphalein dye retention	24	42.7	28.2	5.7	0	0	22	36.1	14.5	3	0	2
Cephalin cholesterol flocculation	16	23.9	23.3	5.8	5	3	19	19	12.7	2.9	4	1
Thymol turbidity pH 7.8	18	29.1	21.5	5.1	4	2	14	30.5	12.5	3.4	4	6

A = Patients with abnormal values which became normal during period of observation.
B = ” ” normal values during entire period of observation.
C = ” ” abnormal ” ” ” ” ” ” .

TABLE II. Comparison of Course of Liver Function Tests in 10 Patients Treated with Chloromycetin and 10 Control Patients.

	Chloromycetin treated						Control group					
		Days after start of therapy when values became normal						Days after start of therapy when values became normal				
Liver function tests	A No. of pt	Mean $\bar{x}$	σ	$\sigma_{\bar{x}}$	B No. of pt	C No. of pt	A No. of pt	Mean $\bar{x}$	σ	$\sigma_{\bar{x}}$	B No. of pt	C No. of pt
Total serum bilirubin	10	30.1	22.1	7	0	0	9	23.9	14.8	4.9	1	0
Bromsulphalein dye retention	10	38.4	18.1	5.7	0	0	9*	46	21	6.6	0	1
Cephalin cholesterol flocculation	9	24.6	16.3	5.5	1	0	8	22.5	15.8	5.6	0	2
Thymol turbidity pH 7.8	7	25.7	12.8	4.8	1	2	9	23.5	12.5	4.1	0	1

* One patient had BSP value of 6.5% at 87 days after start of placebo and was sent to a convalescent hospital for 3 wk, after which the BSP was 6%.

A = Patients with abnormal values which became normal during period of observation.
B = ” ” normal values during entire period of observation.
C = ” ” abnormal ” ” ” ” ” ” .

level.

Effect of combined intravenous and oral therapy with aureomycin. Four patients received aureomycin both orally and intravenously. On admission, these patients were mildly icteric, the highest total serum bilirubin being 5.8 mg %. Although the small number of patients makes it difficult to appraise the effect of combined therapy, no remarkable benefit was noted. The courses of disease of these patients did not differ noticeably from those of the patients treated orally with aureomycin or from the control patients.

Untoward effects of antibiotics. The gastrointestinal symptoms associated with the use of these antibiotics were difficult to appraise in this study because of the prominence of such symptoms in viral hepatitis. However, there was apparently some accentuation of anorexia immediately after the ingestion of aureomycin. Two patients who received combined oral and intravenous therapy with aureomycin developed cheilosis and stomatitis 5 and 7 days after beginning treatment. One of these patients had a red papular rash on the trunk at the same time, which disappeared

TABLE III. Comparison of Course of Liver Function Tests in 20 Patients Treated with Terramycin and 20 Control Patients.

	Terramycin treated						Control group					
		Days after start of therapy when values became normal						Days after start of therapy when values became normal				
Liver function tests	A No. of pt	Mean $\bar{x}$	σ	$\sigma_{\bar{x}}$	B No. of pt	C No. of pt	A No. of pt	Mean $\bar{x}$	σ	$\sigma_{\bar{x}}$	B No. of pt	C No. of pt
Total serum bilirubin	20	22.7	16.4	3.7	0	0	20	35.2	19.6	4.4	0	0
Bromsulphalein dye retention	19	44.1	17.6	4	0	1	20	61	27.8	6	0	0
Cephalin cholesterol flocculation	19	21.9	16.6	3.8	1	0	19	28.4	22.7	5.2	0	1
Thymol turbidity pH 7.8	12	27.3	20.7	6	2	6	16	40	21.3	5.3	1	3

A = Patients with abnormal values which became normal during period of observation.
B = ” ” normal values during entire period of observation.
C = ” ” abnormal ” ” ” ” ” ”.

one day after cessation of treatment. Marked improvement was noted in the stomatitis within a week after discontinuing therapy although the cheilosis continued for a month.

Comment. During the course of the investigation of these antibiotics in the management of viral hepatitis, no immediate or remarkable results were observed. The investigation of aureomycin and chloromycetin revealed minor differences between the treated patients and their respective controls, and statistical analysis of these differences revealed a t value of less than 2; therefore, the differences observed were not statistically significant. A comment is required, however, concerning the apparent difference between the course of the terramycin treated group and that of the terramycin control patients (Table III). In reviewing the treated patients with the control patients in order to determine the comparability of the two groups before treatment was instituted, the average age of the terramycin treated patients was found to be 22.8 years; the day of disease on which treatment was started was 9.04; the mean initial serum bilirubin was 5.23. The control patients were quite similar. The average age was 24.4; the day of disease on which placebo was instituted was 9.06, and the mean initial serum bilirubin was 5.9. In neither group was there a patient who demonstrated confusion, coma, ascites, or edema. As mentioned before, four terramycin control patients showed a maximum serum bilirubin after placebo was started which was over 15 mg %. Only one treated patient had such a high serum bilirubin. After discarding these patients in the analysis, the difference between the two groups still remained significant at less than the 5% level. In reviewing the course of all patients in this study, it can be seen from the tables that the terramycin control patients had a longer course than any other group. However, it should be pointed out that the terramycin study was performed about one year after the investigation of aureomycin and chloromycetin. This renders comparison between the terramycin study and the other antibiotic studies of questionable validity. This study was limited by the clinical material to patients only moderately ill and who had reached the icteric phase of the disease before treatment. It was not possible to evaluate the benefit of aureomycin in hepatic coma(1). Furthermore, in a study of this kind it is obvious that no comment can be made as to the prophylactic value of aureomycin, chloromycetin, or terramycin in this disease. The known presence of serum hepatitis in American troops in Germany(2) makes it probable that both serum hepatitis and infectious hepatitis were under observation in this study.

Summary. The usual course of acute viral hepatitis in young adult men was not significantly changed by the use of aureomycin or chloromycetin. Although terramycin administration was associated with a more rapid return to normal of liver function tests than observed in a control group, further investigation would be required to determine the significance of this observation.

1. Farquhar, J. D., Stokes, J., Jr., Whitlock, C. M., Bluemle, L. W., Jr., Gembescia, J. M., *Am. J. Med. Sci.*, 1950, v220, 166.

2. Evans, A. S., Proc. Soc. Exp. Biol. and Med., 1950, v75, 809.

Received October 9, 1951. P.S.E.B.M., 1952, v79.

Toxicity of Radioiodine and Radiophosphorus in Rats in Doses Comparable To Those Used Clinically.* (19371)

Campbell Moses, Joseph B. Boatman, Robert S. George, and Agatha M. DeLacio.

From the Addison H. Gibson Laboratory, School of Medicine, University of Pittsburgh

The widespread use of radiophosphorus (P^{32}) and radioiodine (I^{131}) in clinical practice emphasizes the need for additional data on the toxicity of these isotopes. Except for the monograph of Bloom(1) and other recent reports(2-11) on the distribution and toxicity of these isotopes little information is available on the toxic effects of these substances in dosages used clinically. These experiments were designed to determine the effects of P^{32} and I^{131} administered in doses comparable to those commonly used therapeutically on the growth and histological appearance of the tissues of young albino rats.

Methods. Male rats of the Sprague-Dawley strain initially weighing approximately 100 g were housed, fed and watered under identical conditions during this study. Three groups consisting of 15 rats each received radioactive phosphorus (P^{32}) in doses of 0.066 microcuries per g (Group I-A), 0.100 μc per g (Group I-B), and 0.166 μc per g (Group I-C). Radioiodine (I^{131}) was administered to 3 similar groups in doses of 0.2 μc per g (Group II-C). Ten rats, receiving similar volumes of distilled water, were used as controls (Group III). These doses are comparable to a dose of radioactive phosphorus in a 70 kg man of 4.6 mc (I-A), 7 mc (I-B), and 11.6 mc (I-C), or of radioiodine of 14 mc (II-A), 42 mc (II-B), and 420 mc (II-C). The isotopes were administered by stomach tube in from 5 to 10 ml of solution in a single dose. All animals were weighed 3 times weekly and red and white blood counts and hemoglobin estimations were made at weekly intervals. Six weeks after the administration of the isotopes the rats were sacrificed by decapitation. Terminal blood counts were taken and the total body weight, the liver, kidney and testicular weight were recorded. Aliquots of these tissues were taken for histologic study.

P^{32} administration. The effects of the administration of P^{32} are indicated in Table I. The average body weights in grams were comparable for both the treated (Group I-A, I-B, I-C) and the control (Group III) animals. Table I indicates that there was a slight decrease in the average weight of the liver, kidney and testes of the treated animals over the controls, but expressed in terms of percentage of total body weight, these, with the exception of the testes, were not statistically different from the control animals.

It was noted that especially after the larger doses (Group I-B, I-C) there was a temporary decrease in the total white count but this had returned to control values within 6 weeks. The effect on the red blood count and hemoglobin was not significantly different in the treated and the control groups. Differential blood counts at the time of autopsy revealed a moderate lymphopenia in the treated animals but no other consistent change. Histologic examination of the kidneys, liver, adrenals, and testes failed to reveal any significant

---

* This study was made possible by a grant from the Damon Runyon Memorial Fund.

TABLE I. Mean Weights 6 Weeks After Radioisotope Administration.

Group	Dose per g body wt	No. rats	Initial wt, g	Final wt, g	Liver, g	Kidneys (2), g	Testes (2), g
	P^{32}						
IA	.066 μc	15	99	295	11.5	2.4	3.3
	% body wt				3.9	.81	1.11
IB	.100 μc	15	103	325	11.5	2.4	3.2
	% body wt				3.5	.74	.97
IC	.166 μc	15	101	286	11.1	2.4	3.3
	% body wt				3.9	.82	1.13
	I^{131}						
IIA	.200 μc	15	99	280	11.9	2.5	3.1
	% body wt				4.3	.90	1.12
IIB	.600 μc	15	99	311	12.2	2.5	3.2
	% body wt				3.9	.82	1.01
IIC	6.000 μc	15	101	314	12	2.5	3.3
	% body wt				3.8	.78	1.01
III	Control	10	101	324	12.5	2.5	3.4
	% body wt				3.9	.77	1.21

or characteristic changes in any of the groups of animals receiving P^{32}.

I^{131} administration. Weight gain was not altered in these animals in the 6 weeks following I^{131} administration. At sacrifice it was found the percentages of body weight of the liver and kidney of the treated animals were smaller than those of the control animals. In this group the testes represented a larger percentage of body weight (Table I). The adrenals and pituitary also weighed less in the treated than the control animals.

In all groups the hemoglobin remained depressed after I^{131} administration returning to the level of the control group at the 4th week. White cell counts remained essentially the same as the control animals throughout the study. Differential blood counts at sacrifice showed an increase of polychromatophils and a slight degree of lymphopenia compared to the control animals. Microscopically, the liver, kidney, adrenals and testes from the 3 groups did not differ from the controls.

Summary and conclusions. 1. Radioactive phosphorus (P^{32}) was administered by intubation to young male rats in doses equivalent to 4.6 mc, 7.0 mc and 11.6 mc for a 70 kg man. Similar radioiodine doses of I^{131} equivalent to 14.0 mc, 42, and 420 mc were given. All animals were sacrificed 6 weeks after dosing. 2. Except for a significant decrease in the testes/body weight ratio and a moderate lymphopenia at the time of autopsy no growth curve, organ weight or histologic evidence of damage was discerned in the animals receiving P^{32}. 3. Although no changes in weight gain or histologic evidence of organ damage was observed following the administration of I^{131}, some variations in organ weight/body weight ratio were recorded. 4. In the doses here employed, which were comparable to those used clinically, no evidence of serious radiation damage was recorded.

1. Bloom, Wm., *Histopathology of Irradiation from External and Internal Sources,* McGraw-Hill, Inc., 1948.

2. Kurland, G. S., Chamovitz, D. D., and Freedburg, A. S., *J. Clin. Invest.,* 1950, v29, 829.

3. Findlay, Leblond, *Am. J. Roent. and Rad. Ther.,* 1948, v59, 389.

4. Warren, Shields, *Cancer Research,* 1943, v3, 334.

5. Platt, Wm. R., *Arch. Path.,* 1947, v43, 1.

6. Henshaw, P. S., Snider, R. S. and Riley, E. F., *Radiology,* 1949, v52, 401.

7. Gorbman, Aubrey, PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 237.

8. Cornatzer, W. E., Harrell, G. T., Cayer, D. and Antrom, C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 492.

9. Bonner, J., *Fed. Proc.,* 1950, v9, 328.

10. Warren, S., MacMillan, J. C. and Dixon, F. J., *Am. J. Roent. and Rad. Ther.,* 1950, v55, 375 and 557.

11. Gorbman, A., *J. Clin. Endocrin.,* 1950, v10, 1177.

Received December 7, 1951. P.S.E.B.M., 1952, v79.

Tissue Metabolism in Growing Birds.* (19372)

RICHARD R. CRANDALL AND ARTHUR H. SMITH. (Introduced by V. S. Asmundson.)
From the College of Agriculture, University of California, Davis.

Recently Bertalanffy and Pirozynski(1) have studied the metabolic rates of tissues from growing rats *in vitro* and related them to body size. They found a general decrease in oxygen consumption with increasing size which was less than would have been expected on the basis of "surface law" or "metabolic size." These results seem to indicate a lack of correlation between the metabolic rate of the whole animal and of its tissues *in vitro*.

In order to investigate the relation between *in vitro* tissue and whole animal metabolic rate, we have carried out tissue respiration trials with tissues from chickens of different age. Whereas rats have a high metabolic rate per unit weight shortly after birth which decreases with increasing age (Kleiber, unpublished), chickens have a low metabolic rate per unit weight after hatching, which increases during the first month(2). This contrast in metabolic rate in growing animals of these two species makes a comparison of their metabolism *in vitro* of interest to the general problem of body size and metabolic rate(3).

Method. Chickens were selected to form several age groups which would approximate the critical points on the basal metabolic rate curve. These groups were: Group I, 5-17 days, males; Group II, 27-34 days, males; Group III, mature laying females. Groups I and II were fasted 12-20 hours prior to the experiment at a controlled temperature of 33°C and Group III at 30°C. Oxygen consumption rates of slices and homogenates of liver and kidney were determined *in vitro* by Warburg's direct method(4). These measurements were made at 39.8 ± .2°C, which approximates the body temperature of birds.

Results. The oxygen consumption rate (cu mm, n.t.p. per hour) per mg dry tissue (Qo_2) for slices and homogenates was calculated for each bird. A summary of each group is given in Table I.

Discussion. In liver slices from a wide variety of mature mammals, Kleiber(5) derived the following regression equation relating oxygen consumption rate *in vitro* (Qo_2) to body weight (kg): $Qo_2 = 5.43\ W^{-.21}$. When the liver slice data for mature hens is calculated to the same units of body weight, the relationship is close to that found in mammals: $Qo_2 = 6.41\ W^{-.21}$. It is also known that the metabolic rate of the whole mature chicken (and birds in general) fits the weight-metabolism curve established among mature mammals(6). Thus, the relationship between liver slice and whole animal metabolic rate per unit weight in mature chickens is the same as that in mature mammals.

When the oxygen consumption rates of the bird kidney slices are compared with those of mammals(7) it is obvious that they are much lower regardless of body size. This may result from a lesser ability of the chicken kidney to do osmotic work(8).

An intraspecific comparison of the tissue metabolic rate with that of the whole animal (Table I) in growing animals is quite different for the liver and kidney. Kidney tissue respiration (both slice and homogenate) is not significantly different between any of the groups studied. Apparently the metabolic rate of this tissue *in vitro* is independent of body size or whole animal metabolism; this seems generally to be the case in other species (1,7).

Liver tissue from chickens of different ages presented quite a variation in oxygen consumption rate. The liver homogenate Qo_2 of the very young (Group I) and mature (Group III) birds was about the same, that of the one month birds (Group II) was significantly greater than either. The liver slices of the one-month-old birds was also significantly greater than that of the mature birds. Although these changes in tissue metabolic rate are in the same direction as the changes in whole animal metabolic rate of birds of

---

* The authors gratefully acknowledge the technical assistance of Robert M. Bramman.

TABLE I. Tissue and Whole Animal Basal Metabolic Rate of Chickens.

Group	Age (days)	No. per group	Body wt (g)	Liver Dry (%)	Liver Qo_2* Slice	Liver Qo_2* Homog.	Kidney Dry (%)	Kidney Qo_2* Slice	Kidney Qo_2* Homog.	kCal/day per:† kg	kCal/day per:† kg ¾	kCal/day per:† kg ⅔
I Chicks	5-17	16	66 ± 4	26.7 ± .3	—	1.61 ±.17	23.2 ±.4	—	1.52 ±.06	173	79	61
II Chicks	27-34	7	178 ± 12	25.6 ± .5	6.71 ±.42	4.31 ±.47	22.5 ±.3	10.71 ±.96	1.49 ±.14	201	110	88
III Hens	Mature	6	2350 ± 230	31.1 ±2.6	5.44 ±.28	1.88 ±.27	22.4 ±.3	9.22 ±.49	1.41 ±.17	73	87	93

* $Qo_2 = mm^3\ O_2$ (ntp) consumed per hr per mg dry tissue.

† Whole animal basal metabolic rate; groups I and II, Kleiber(2); group III laying hen, Mitchell and Haines(9).

these ages, the curves are not parallel. The ratio: $\frac{\text{liver slice metabolic rate}}{\text{whole animal metabolic rate per unit wt}}$ increases in the older animals (proportional to the cube root of the body weight).

Conclusions. 1. Tissue respiration studies show that oxygen consumption rate of mature chicken liver slices fits body weight-tissue metabolic rate relationship derived by Kleiber (5) for mature mammals. The oxygen consumption rate of mature chicken kidney slices, however, is much lower than that of mature mammals(7). 2. The relationship between tissue and whole animal metabolic rate in growing chickens is different for the kidney and liver. The Qo_2 of kidney tissue (homogenate or slice) is essentially the same for all age groups of birds tested. The Qo_2 of liver tissue *in vitro* is the same for very young and mature birds, but is significantly higher in one-month-old birds. This same sequence has been noted by Kleiber(2) in the whole animal metabolic rate (per unit weight) of growing birds.

---

1. Bertalanffy, T. von, and Piroxzynski, W. J., *Science*, 1951, v113, 599.

2. Kleiber, M., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 793.

3. Kleiber, M., *Physiol. Rev.*, 1947, v27, 511.

4. Dixon, M., *Manometric Methods as Applied to the Measurement of Cell Respiration and other Processes*, 2nd ed., Macmillan, New York, 1943.

5. Kleiber, M., PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 419.

6. ———, *Hilgardia*, 1932, v6, 315.

7. Krebs, H. A., *Biochim et Biophys. Acta*, 1950, v4, 249.

8. Korr, I., *J. Cell. Comp. Physiol.*, 1939, v13, 175.

9. Mitchell, H. H., and Haines, W. T., *J. Agr. Res.*, 1927, v34, 927.

Received December 13, 1951. P.S.E.B.M., 1952, v79.

Survival of Thermally Injured Rats Infused with Saline, Polyvinylpyrrolidone, Dextran and Oxypolygelatin. (19373)

MILES D. MCCARTHY AND JERRY W. DRAHEIM. (Introduced by I. S. Ravdin.)

From the Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania, Philadelphia.

Using the standardized back burn procedure developed in this laboratory for the white rat (1), we have obtained additional information which demonstrates that intravenous NaCl solutions are effective in combating burn shock and promoting survival of these animals. Macromolecular solutions* of polyvinylpyrrolidone (P.V.P.), Dextran, and Oxypolygelatin (O.P.G.) have been tested simultaneously with the saline solutions and the macro-

---

* Allocated by the National Research Council, Subcommittee on Shock. (P.V.P. Schering-Batch GA-P.V.P.-111; Dextran C.S.C. Lot 84665A; O.P.G. Don Baxter S152AY).

TABLE I. Postburn Survival Rates for Various Infused Groups of Rats Burned at 90°C for 35 Seconds, 32 ± 2% Total Body Surface.

	Infused with 10% body wt postburn: 4%, 2 hr; 3%, 5 hr; 3%, 10 hr				Infused with 4% body wt postburn: 2%, 2 hr; 2%, 5 hr		
		% survival, postburn				% survival, postburn	
Infusion fluid, %	No. rats	8 hr	24 hr	10 days	No. rats	8 hr	12 hr
Untreated controls	40	20	0	0			
NaCl 1.4	20	80	80	75			
1.2	20	70	70	70	6	50	0
.9	20	80	70	60	6	0	0
P.V.P. .875	20	95	65	65			
1.75	9	89	33	33			
3.5	12	50	33	33	6	50	0
4.5	12	58	8	8			
O.P.G. 5	12	50	33	25	6	17	0
Dextran 1.5	6	83	0	0			
6	6	66	0	0			

molecular solutions have been found to be less efficacious in promoting 10-day survival than the saline solutions. The latter were found to show greater efficacy as the percentage of NaCl was increased from .9% to 1.2% and 1.4% respectively.

Material and methods. White rats of the Wistar strain, weighing between 190 and 210 g, have been used as the test animals. All rats received a back burn under ether anesthesia of 32 ± 2% of the total body surface and the experiments were usually conducted on 12 rats at a time. Prior to burning, the animals were segregated at random into several equal groups to permit testing of 2 or more infusion fluids simultaneously along with an untreated control group. Following the burn all rats were placed in individual wire mesh cages. Infusions were made at the rate of approximately 1 cc per minute through a small skin incision into the saphenous vein at postburn intervals of 2 hours, 5 hours, and 10 hours. Food and water were withheld from all animals for a period of 12 hours postburn. After this interval food and water were offered *ad lib.* Survival rates were recorded at the following postburn intervals: 8, 12, 24, 48, 72 hours and 10 days. The macromolecular materials were all suspended in isotonic saline. Hematocrit samples were obtained as previously described(2) from the lateral tail vein of several animals in each infused group.

Results. Table I shows the survival percentages for rats receiving the several different infusion fluids administered as 10% of the body weight and as 4% of the body weight. These data demonstrate that a) a 10% infusion volume is necessary for survival, b) hypertonic saline solutions are more effective in promoting survival than isotonic solutions, c) solutions of increasing concentrations of the macromolecular substances become less effective in promoting survival.

A specific type of anaphylactic-like reaction was noted in the Dextran infused rats which was much less pronounced than similar reactions following Dextran infusions in normal animals(3). Thus, the rat proved to be a relatively unsuitable species in which to test Dextran and further testing of this material was discontinued.

Hematocrit data presented in Table II demonstrate that the infused solutions of 1.4% NaCl produced a greater hemodilution followed by less hemoconcentration than 0.9% NaCl solutions. Greater hemodilution followed macromolecular infusions and hemodilution increased as the concentration of the P.V.P. solutions increased. Hemoconcentration was markedly reduced by infusions of 4% of the body weight of 3.5% P.V.P. solutions. Rats so treated all died, even though the hematocrit values of such rats were within the range of those of animals which survived following 10% infusion volumes.

Discussion. Earlier work in this laboratory

TABLE II. Average Preburn Hematocrit Values and Average Postburn Hematocrit Values as % of Preburn Hematocrits for Several Rats from Each Infused Group in Table I. Hematocrits taken just before infusion and 5 min postinfusion at the following postburn intervals.

Infusion fluid, %	Preburn	Postburn						
		2 hr		5 hr		10 hr		
		Pre	Post	Pre	Post	Pre	Post	24 hr
I. Two hr (4% body wt infused), 5 hr (3% body wt infused), 10 hr (3% body wt infused).								
Untreated*	45.5(18)	115.5(18)	—	122.6(13)	—	Dead	—	—
NaCl 1.4	43.6 (7)	120.6 (7)	98.9 (7)	114.2 (5)	96.95(6)	101.7 (4)	76.8 (4)	74.5 (4)
1.2	42.5 (7)	123.9 (7)	97.8 (6)	120.1 (7)	100.6 (7)	109.2 (5)	85.7 (5)	79 (5)
.9	45.9 (7)	117.5 (7)	105.6 (7)	120.1 (7)	103.8 (6)	99.7 (4)	89.4 (4)	76.5 (3)
P.V.P. .875	45.1 (7)	116.9 (7)	79.8 (7)	109 (6)	85.2 (6)	101.6 (4)	70.5 (4)	89.2 (4)
1.75	43.1 (3)	116.1 (3)	84.1 (3)	115.1 (3)	84.4 (3)	107.4 (1)	74.2 (1)	72 (1)
3.5	45 (5)	122 (5)	64.5 (5)	102.7 (5)	68.4 (4)	Dead	—	—
4.5	43.8 (5)	111.5 (4)	60.3 (5)	99.1 (5)	59.5 (5)	104.7 (2)	70 (2)	Dead
O.P.G. 5	45.1 (6)	112.3 (6)	64.2 (6)	105.5 (5)	72.7 (5)	116.8 (1)	Dead	—
Dextran 1.5	44.7 (3)	119.7 (3)	107.7 (3)	112 (3)	74 (3)	Dead	—	—
6	41.6 (3)	116.6 (3)	94 (3)	113.6 (3)	57.8 (3)	Dead	—	—
II. Two hr (2% body wt infused), 5 hr (2% body wt infused).								
NaCl 1.2	44.6 (3)	119.5 (3)	117 (3)	116.5 (2)	106.8 (2)	Dead		—
.9	44 (3)	131.3 (3)	111.2 (3)	130.2 (2)	115.5 (1)	Dead		—
P.V.P. 3.5	42.6 (3)	123.1 (3)	79.7 (3)	108.4 (2)	79.7 (2)	Dead		—
O.P.G. 5	45.7 (3)	124.6 (3)	105.2 (3)	107.1 (1)	Dead	—		—

* Number in parentheses after each percentage indicates number of rats studied. Except for an occasional lost value, a change in these numbers indicates death of one or more animals.

(2) had indicated that a) this burn procedure was 100% lethal to untreated animals, b) infusion volumes of 10% of the body weight were necessary to promote survival, c) hypertonic saline (1.06%) solutions were more efficacious in promoting survival than normal physiological saline solutions. Consequently, in most of these experiments 10% of the body weight was infused in 3 stages postburn, 4% at 2 hours, 3% at 5 hours and 3% at 10 hours. In several experiments 4% of the body weight was infused in 2 equal volumes of 2% each at a 2- and 5-hour postburn interval. Accordingly, NaCl solutions of the following compositions were infused: 0.9%, 1.2% and 1.4%.

It should be kept in mind that the survival percentages recorded in this paper are not absolute, but represent relative positions with respect to efficacy in promoting survival. It has been our experience that many physical factors influence survival of severely burned rats, among these are handling of the animals and the season of the year. This latter factor is in part a temperature and humidity effect. The experiments reported herein were conducted between July 15 and October 1, during which period there was a room temperature range of 24.3-30.8°C, average 27.1°C; and a relative humidity range of 47-79%, average 63.7%. Survival percentages of all groups would be expected to show corresponding increases if similar experiments were conducted during the winter months.

The results of these experiments are in general agreement with those of Rosenthal (4); namely, that sodium chloride causes a significant reduction in deaths following burns, and that the majority of deaths occur within the first 24 hours following the burn. In several respects, however, these data are at variance with Rosenthal's findings. In our experience hypertonic saline administered by the systemic route promotes higher survival rates than 0.9% saline similarly administered, and the 10-day survival period indicated that the majority of rats which survived 24 hours, continued to do so.

Summary. 1. Ten-day survival percentages are recorded for severely burned rats, infused with saline and several macromolecular solutions and for the simultaneous untreated controls. a) Hypertonic saline solutions were more effective in promoting survival than isotonic saline solutions. b) Effectiveness of the macromolecular solutions in promoting survival decreased as the concentration of

these substances was increased. c) Due to the specific allergic reaction of rats to Dextran, the relative effectiveness of this plasma expander could not be ascertained. 2. Hematocrit data recorded in terms of per cent of initial values are given for several animals in all groups. a) Hypertonic saline solutions tend to maintain a cell-plasma ratio more nearly approaching the initial than isotonic solutions. b) Increased hemodilution followed infusions of the higher concentrations of macromolecular substances. c) Hemodilution in general cannot be directly correlated with survival; however, the lowest hemodilution values were found, except for saline, in those groups having the lowest survival percentage.

1. McCarthy, M. D., *J. Lab. and Clin. Med.*, 1945, v30, 1027.

2. McCarthy, M. D., Parkins, W. M., *Am. J. Physiol.*, 1947, v150, 428.

3. Voorhees, A. B., Baker, H. J., and Pulaski, E. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 254.

4. Rosenthal, S. M., *Public Health Rep.*, 1943, v58, 513.

Received December 27, 1951. P.S.E.B.M., 1952, v79.

Cyclophorase System, XXII. Cyclophorase System of Rabbit Heart Muscle.* (19374)

MILTON H. PAUL,† M. FULD,‡ AND E. SPERLING. (Introduced by P. W. Wilson.)
From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisc.

Previous studies in this laboratory have dealt with the reactions involved in the complete oxidation of pyruvic acid, fatty acids and certain amino acids by the cyclophorase-mitochondrial (CM)§ system of rabbit liver and kidney(1-6). The present report deals with some properties of the CM system of rabbit heart muscle. The heart system (cat) has been studied by Ochoa(7) with special reference to the oxidation of α-ketoglutarate, and (in the rat) by Lehninger(8) and Potter (9) with reference to fatty acid oxidation and oxidative phosphorylation, respectively. From the standpoint of systematics, it is important to know how the CM system of heart muscle compares with those of kidney and liver with respect to properties and metabolic spectrum. The present report deals with some comparative studies along these lines.

Preparation of the CM system. The heart (obtained immediately upon sacrifice of the animal) is divested of its pericardial sac and auricular tissue, and packed in ice (average weight, 5 g). The iced, ventricular tissue is cut into small pieces, added directly to 75 ml of 0.9% potassium chloride (0°C) in the small, monel metal, Waring blendor and blendorized for one minute. The resultant suspension is passed through a double layer of gauze to remove fibrous tissue and then centrifuged at 3-5°C for 7 minutes at 2500 x g. After discarding the supernatant fluid, the residue (R_1H) is resuspended in cold 0.9% potassium chloride and centrifuged again for 5 minutes. The residue (R_2H) is now made up to 6 ml with 0.9% potassium chloride. The Q_{O_2}¶ of these preparations range from 33-45. The R_2H accounts for about 40% of the oxidative activity of the original homogenate. It was found in some but not all cases that when the R_2H is supplemented with the supernatant of the first centrifugation, the original oxidative activity can be reached. Phase microscopy of the preparation disclosed nuclei and nuclear fragments in addition to mito-

---

* This investigation was supported by a grant from the Heart Institute of the National Institutes of Health.

† Present address: Army Medical Center, Washington, D.C.

‡ Present address: Graduate School of Public Health, University of Pittsburgh.

§ For the association of cyclophorase activity with mitochondria *cf.* Harmon, *J. Exp. Cell Res.*, 1950, v1, 382.

¶ μl oxygen absorbed/hr/mg dry wt of preparation in presence of α-ketoglutarate at 38°C.

TABLE I. Effect of Cofactors.

	μl O_2 30 min	μl O_2 120 min
A. Complete system	1034	
no phosphate	357	
no adenosine-5-phosphate	129	
no substrate	0	
B. Complete system	650	1300
+ diphosphopyridine nucleotide (500 γ)	602	1250
+ cocarboxylase (200 γ)	600	1320
+ flavindinucleotide (60 γ)	580	1280
+ cytochrome c (350 γ)	650	1300

The complete system contained 1 ml of enzyme preparation, .2 ml of .1 M phosphate buffer, pH 7.4, .3 ml of .01 M adenosine-5-phosphate, .2 ml of .1 M $MgCl_2$ and 20 μ M α-ketoglutarate. Final volume 3 ml 38°C, oxygen in gas phase.

TABLE II. Specificity of the Adenine Nucleotide.

	μl O_2 80 min
System without adenine nucleotide	160
+ .3 ml .01 M adenosine-5-phosphate	1260
+ .2 ml .025 M adenosinediphosphate	1335
+ .1 ml .05 M adenosinetriphosphate	1333

The complete system contained 1 ml enzyme preparation, .2 ml of .1 M phosphate buffer, pH 7.4, .2 ml of .1 M $MgCl_2$ and 20 μM α-ketoglutarate.

chondria. A few intact myofibrils were also recognizable.

Methods. In most experiments the substrate was mixed with the enzyme system before the manometers were placed in the bath. This was necessary, since in absence of substrate, the enzyme system deteriorates rapidly. After a 5-minute equilibration period, the taps were closed. The oxygen uptake was corrected to include the equilibration period on the basis of the rate recorded during the first 5-minute period. The experiments on oxidative phosphorylation were carried out at 25°C. Inorganic phosphate was determined by both the Fiske-Subbarow(10) and Lowry-Lopez methods(11) with excellent agreement between the two methods.

Results. Components for maximum activity. Only 3 components are essential for maximum activity, *viz.* inorganic phosphate, magnesium ions and adenosine-5-phosphate (Table I, A). These requirements were shown previously by Ochoa(7) to apply to the CM system of cat heart muscle; in our own laboratory the same requirements were shown to apply to the CM system of rabbit liver and kidney. The addition of cytochrome *c*, diphosphopyridinenucleotide, cocarboxylase or flavinadenine dinucleotide had no effect either on the initial rate of oxidation or on the rate of decline of activity (Table I, B). The CM system of rabbit heart muscle as prepared above, shows a rapid decline in activity after the first 30 minutes. By contrast, the CM systems of rabbit liver and kidney show linear rates of oxidation for 1-2 hours. Adenosinemonophosphate can be replaced by either adenosinediphosphate or adenosinetriphosphate (Table II). The optimum levels of adenosine-5-phosphate and inorganic phosphate are the same in the heart system as for other systems. However, the optimum level of magnesium ions is about 5 times that described for the liver and kidney system. This requirement for higher magnesium levels is clearly demonstrable in a well-washed enzyme preparation (Table III), or in an aged enzyme preparation (Fig. 1). The addition of magnesium chloride to a fresh preparation (R_2H) produces little stimulation—whereas a preparation which has been aged for 4 hours at 0°C and then washed with saline solution, shows the full original activity only when supplemented with the higher level of magnesium chloride.

Metabolic spectrum. The diagnostic which we have used for an intact CM system is the ability to carry out the full citric acid cycle. The data of Table IV establish that the CM system of rabbit heart muscle catalyzes the complete oxidation of each of the intermediates of the citric acid cycle. With respect to

TABLE III. Magnesium Requirements.

	μl O_2 30 min
I. R_2H—no $MgCl_2$	452
+ .2 ml of .1 M $MgCl_2$	515
II. R_3H—no $MgCl_2$	164
+ .2 ml of .1 M $MgCl_2$	505
III. R_3H (centrifugation carried out with .9% KCl .0013 M $MgCl_2$)	522

The complete system contained 1.5 ml enzyme preparation, .2 ml .1 M phosphate buffer, pH 7.4, .3 ml .01 M adenosine-5-phosphate and 20 μM α-ketoglutarate.

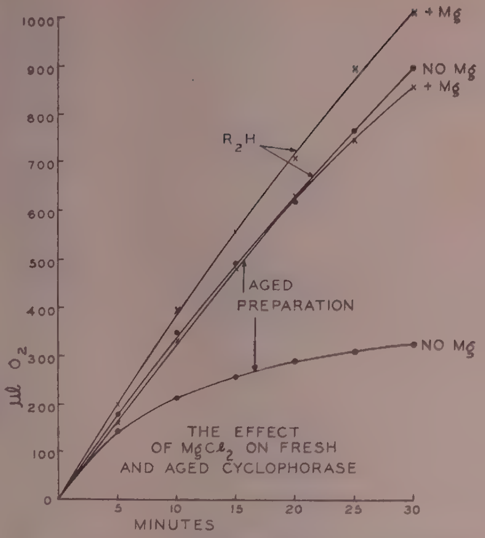


FIG. 1. Effect of magnesium ions in fresh and aged rabbit heart muscle CM preparations. The fresh preparation was brought to the stage of R_2H. The aged (4 hr) preparation was washed with .9% potassium chloride and brought to the R_3H stage. Details as in legend for Table III.

fatty acid oxidation,‖ the pattern is similar to that for the kidney system. According to manometric analyses (Table V), even-numbered fatty acids—including acetoacetate—are oxidized to carbon dioxide and water, whereas odd-numbered fatty acids are oxidized to carbon dioxide, water, and propionate. A well-washed enzyme preparation (R_3H) requires the presence of small amounts (1-2 μ moles) of some member of the citric acid cycle to initiate fatty acid oxidation. The oxidation of fatty acids is completely inhibited by 2:4-dinitrophenol at a level of 6.5×10^{-6} M (Table VI).

The oxidation of L-amino acids by the rabbit heart CM preparation has not yet been thoroughly investigated. L-glutamate is oxidized consistently at a rapid rate. Repeated attempts to demonstrate manometrically oxidation of lactate in the heart preparation were unsuccessful with or without supplements, such as diphosphopyridinenucleotide or the supernatant from the first centrifugation. Some lactic dehydrogenase activity could be detected by the anaerobic Thunberg technic.

Coupling of oxidation and phosphorylation. Ochoa(7), using cat heart muscle preparations, and Cross *et al.*(12), using rabbit liver and kidney CM preparations, have shown that esterification of inorganic phosphate accompanies all the oxidative steps of the citric acid cycle with an overall P/O ratio of about 3. Table VII shows a typical experiment with the CM system of rabbit heart muscle in which an average P/O ratio of 2.9 is attained.

Discussion. The CM system of rabbit heart muscle appears to share the basic properties of mitochondrial systems generally.

TABLE IV.
Oxidation of Citric Acid Cycle Substrates.

Substrate	μM added	Oxygen utilized, μatoms Observed	 Theory
Pyruvate	16	80.7	80
α-Ketoglutarate	5	42.6	40
Succinate	5	34.8	35
1-Malate	5	29.7	30
Fumarate	5	32.6	30
Oxalacetate	3	14.9	15
Citrate	4	36	36

The complete system contained 1.5 ml enzyme preparation, .2 ml of .1 M phosphate buffer, pH 7.4, .3 ml of .01 M adenosine-5-phosphate, and .2 ml of .1 M $MgCl_2$.

TABLE V. Oxidation of Fatty Acids.

Substrate	μM	Oxygen utilized, μatoms Observed	 Theory
Acetate	10	41.6	40*
Butyrate	10	115	100*
DL-β-hydroxybutyrate	30	151	135*†
DL-β-hydroxycaproate	10	94	75*†
Acetoacetate	9	70.5	72*
n-caproate	4	64.5	64*
Octanoate	2	43.7	44*
Propionate	20	0	140*
Acrylate	6	0	36*
n-valerate	5	33	30‡
Heptylate	2.5	35.4	30‡
Pelargonate	3.5	59.1	63‡
Δ_{10-11} undecylenate	2	41.2	46‡

* Calculated for oxidation to CO_2 and H_2O.

† Theory for oxidation of only one isomer. For complete system, see Table IV.

‡ Calculated for oxidation to CO_2 and H_2O and propionate.

‖ The demonstration of fatty acid oxidation in CM systems prepared from pig heart, beef heart and pigeon breast muscle has not been as unequivocal as in the case of the CM system of rabbit heart. It has yet to be decided whether the CM systems of cardiac tissue generally can oxidize fatty acids.

TABLE VI. Effect of Dinitrophenol on Fatty Acid Oxidation.

	μatoms O_2 60 min
Complete system	
+ 3 μM succinate	8.3
+ " " + 5 μM caproate	79.9
+ " " + " " + 6.5 × 10^{-6} M 2,4-dinitrophenol	8.3

Complete system as in Table IV.

TABLE VII.
Coupling of Oxidation and Phosphorylation.

Time, min	Inorganic phosphate utilized, μM	O_2 uptake, μatoms	P/O
0- 5	12.9	4.3	3
5-10	12.2	4.3	2.8
10-15	12.2	4.3	2.8

The complete system contained 1 ml enzyme preparation, .2 ml .1 M $MgCl_2$, .3 ml of .01 adenosine-5-phosphate, .4 ml of .1 M phosphate buffer, pH 7.4, .1 ml of .3 M NaF, .1 ml of M glucose, .05 ml of yeast hexokinase and 30 μM pyruvate. 25°C.

The higher level of magnesium requirement is the only unusual feature of the heart preparation. As prepared, the system contains the full complement of enzymes and coenzymes necessary to implement the complete oxidation of members of the citric acid cycle, fatty acid oxidation and oxidative phosphorylation. The rabbit heart system is not as stable under working conditions as the CM system of rabbit kidney. A marked decline in activity at 38°C is usually observed after some 30 minutes. The instability of the working system may be referable either to impurities in the preparation, or to the instability of the mitochondrial unit itself.

Summary. The preparation and some properties of the cyclophorase-mitochondrial system of rabbit muscle are described.

We wish to thank Dr. D. E. Green for his guidance and helpful suggestions.

1. Green, D. E., Loomis, W. F., and Auerbach, V., *J. Biol. Chem.*, 1948, v172, 389.
2. Grafflin, A. L., and Green, D. E., *J. Biol. Chem*, 1948, v176, 95.
3. Still, J. L., Buell, M. V., and Green, D. E., *Arch. Biochem.*, 1950, v26, 406.
4. Still, J. L., Buell, M. V., Knox, W. E., and Green, D. E., *J. Biol. Chem.*, 1949, v179, 831.
5. Still, J. L., Buell, M. V., and Green, D. E., *Arch. Biochem.*, 1950, v26, 413.
6. Taggart, J., and Krakauer, R. B., *J. Biol. Chem.*, 1949, v177, 641.
7. Ochoa, S., *J. Biol. Chem.*, 1944, v155, 87.
8. Lehninger, A. L., *J. Biol. Chem.*, 1946. v165, 131
9. Potter, V. R., *J. Comp. and Cell. Physiol.*, 1945, v26, 87.
10. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem*, 1925, v66, 375; 1929, v81, 629.
11. Lowry, O., and Lopez, J. A., *J. Biol. Chem.*, 1946, v162, 421.
12. Cross, R. J., Covo, G. A., Taggart, J. V., and Green, D. E., *J. Biol. Chem.*, 1949, v177, 655.

Received January 7, 1952. P.S.E.B.M., 1952, v79.

Cyclophorase System XXIII. Correlation of Cyclophorase Activity and Mitochondrial Density in Striated Muscle.[*] (19375)

MILTON H. PAUL[†] AND E. SPERLING. (Introduced by P. W. Wilson.)
From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisc.

Investigations in several laboratories(1-4) have established that mitochondria contain the complete enzymatic apparatus for implementing the reactions of the citric acid cycle and for generating oxidatively high energy phosphate bonds. The functional aspects of mitochondria are covered by the term "cyclophorase activity". Thus, some relation might be anticipated between the physiological function, and either the mitochondrial density or cyclophorase activity of that same tissue.

In this study, crude enzyme preparations were made from muscles of different physiological activity. The oxidative activity was measured manometrically; the mitochondrial density of the same preparations was estimated with aid of the phase microscope.[‡] The ability of the enzyme preparation to oxidize more than one substrate of the citric acid cycle was taken as a diagnostic test for cyclophorase activity. It was necessary in this study to test each enzyme preparation against several substrates simultaneously, since none of the tissues (with the exception of the myocardium(5) and pigeon breast muscle), yielded preparations active enough to oxidize these substrates to completion. The assumption was made that if the same enzyme preparation oxidizes more than one member of the citric acid cycle, the observed oxygen uptake in presence of a given substrate cannot be attributed exclusively to a one-step oxidation.

---

[*] This investigation was supported by a grant from the Heart Institute of the National Institutes of Health.

[†] Present address: Army Medical Service Graduate School, Army Medical Center, Washington, D.C.

Experimental. Preparation of Enzymes. The tissue is blendorized for one minute in a small glass Waring blendor with 10 volumes of cold 0.9% potassium chloride. The suspension during this time is maintained neutral to bromthymol blue by addition of N sodium hydroxide. The tissue suspension is then passed through a double thickness of gauze to remove fibrous tissue, and centrifuged at 0°C for 8 minutes at 2500 x g. The supernatant is discarded, and the residue made up to a thick suspension with the addition of 0.9 percent potassium chloride. This suspension is used directly in the experiments. *Determination of Enzyme Activity.* The Warburg manometric technic was used for estimation of the enzymatic activity. Each manometer flask contained 1 ml of enzyme preparation, 0.2 ml of 0.1 M phosphate buffer of pH 7.4, 0.2 ml of 0.1 M magnesium chloride, 0.3 ml of 0.01 M adenosine-5-phosphate and water to a final volume of 3 ml, alkali in center well and oxygen in gas space. The reaction was carried out at 38°C. *Cyclophorase Quotient.* The standard of comparison in this study is an arbitrary quotient called the "cyclophorase quotient" which provides a basis for comparing the cyclophorase activities of different tissues.

$$\text{Cyclophorase quotient} = \frac{\text{dry wt total cyclophorase preparation (mg)} \times Q_{O_2}\S}{\text{initial wet wt of tissue (g)}}$$

The dry weight of the cyclophorase preparation is calculated from the dry weight of 1 ml. Dry weight determinations (overnight at 110°) were carried out in duplicate. A correction is applied for the salt content of the preparation. The Q_{O_2} is determined on the basis of the rate for the first seven minutes following the initial equilibration period (7 minutes). *Estimation of Mitochondrial Density.* An aliquot of each tissue preparation is diluted 50 or 200 times with isotonic sucrose and examined under oil immersion lens with the phase microscope. Mitochondrial densities are rated as 0 (few or none observed), to $++++$ (maximum number observed). The preparations contain, in addition to mitochondria, both nuclei and nuclear fragments and occasionally a few intact cells.

Results. Table I illustrates the correlation between the physiological activity of a muscle and both the cyclophorase activity and mitochondrial density of the tissue preparation.[¶] The rabbit heart and diaphragm, and the pigeon breast muscle are called upon for strenuous rhythmic and prolonged performance, and accordingly require a vigorous, aerobic, oxidative mechanism. These tissues have been shown to have high cyclophorase quotients and abundant mitochondria. The

---

[‡] Since the technic of mitochondrial counting has yet to be perfected our evaluation of mitochondrial density was only qualitative.

[§] The cyclophorase quotient can serve only as the basis of a qualitative comparison of different tissues. The contribution of non-mitochondrial particulate elements to the dry weight of the residue is not constant from one muscle to another. However, since myosin constitutes the bulk of the residue in all the muscles compared even extreme variations in the level of other particulate elements would not change seriously the order of magnitude of the quotient. Another assumption underlying the quotient is that the activity per mitochondrion is fairly constant regardless of source. Studies in this and other laboratories provide strong support for this assumption. Finally, the conclusions drawn from differences in the values of the quotients are based on variations which are of a different order of magnitude than those which correction factors could account for.

[¶] With exception of experiments on muscles of bat and mallard, all other experiments have been repeated using 3-5 individuals of the animals tested.

TABLE I. Cyclophorase Activity and Mitochondrial Density of Various Muscles.

Tissue	Cyclophorase quotient α-ketoglutarate	Cyclophorase quotient Succinate	Q_{O_2}* α-ketoglutarate	Q_{O_2}* Succinate	Mitochondrial density
Back muscle (white) rabbit	35	75	.5	1.1	0
Breast ” ” chicken	110	110	1.5	1.5	0
Gastrocnemius ” rabbit	100	100	2.7	2.7	0
Soleus (reddish) ”	85	120	3.4	4.7	+
Leg muscles (reddish) rat	85	220	2.5	6.7	+
Forearm muscles (red) bat	185	470	10.2	30.6	++
Diaphragm ” rabbit	545	595	23	25	++
Breast muscle ” mallard	875	1660	11.6	21.9	+++
Heart muscle ” rabbit	1110	1820	34.5	56.7	++++
Breast muscle ” pigeon	1830	2660	24	33	++++
Kidney, rabbit	2150	3080	28	40.3	++++

* Oxygen uptake in μl/hr/mg dry wt of suspension after correction for the salt content.

TABLE II.
Reproducibility of Cyclophorase Quotient.

Preparation	Cyclophorase quotient α-ketoglutarate
1	1110
2	1350
3	1230

muscles which are active only sporadically, such as skeletal muscles, generally have low cyclophorase activity and few mitochondria.

The breast muscle of birds provides a striking illustration of this thesis. In the pigeon (capable of sustained flight), the breast muscle has a cyclophorase activity as high as that of heart muscle and an abundance of mitochondria. In the chicken (limited and occasional flight), the cyclophorase activity of the breast muscle is very low, and there are few or no mitochondrial units which can be recognized by microscopic examination.

The reproducibility of the cyclophorase quotient is indicated in Table II by the results of determinations on three successive heart preparations.

Discussion. The correlation between the physiological activity of various types of striated muscle and their gross and microscopic characteristics(6) have long been emphasized. However, only more recently have the biochemical implications of these differences been considered.

It is remarkable that as far back as 1889, Knoll(7) made the following observations. "The abundance of interstitial granules" (undoubtedly referring to mitochondria in present terminology) "in the active muscles of the pigeon and the heart muscles of all animals points to a correlation between these interstitial granules and the function of the muscle." Kisch(8) has recently presented elegant electron microscope pictures of mitochondria in cardiac tissue.

Summary. The skeletal muscles which are used only sporadically generally have relatively low cyclophorase activity and a low mitochondrial count; whereas muscles which have to function either continuously or for prolonged periods (cardiac muscle, diaphragm, flying muscles), have a high cyclophorase activity and an abundance of mitochondrial units.

We wish to thank Dr. D. E. Green for his guidance and helpful suggestions.

---

1. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1949, v177, 893.
2. Kennedy, E. P., and Lehninger, A. L., *J. Biol Chem.*, 1949, v179, 957.
3. Harman, J. W., *Exp. Cell Research*, 1950, v1, 382.
4. Green, D. E., Loomis, W. F., and Auerbach, V., *J. Biol. Chem.*, 1948, v172, 389.
5. Paul, M. H., Fuld, M., and Sperling, E., This Journal.
6. Needham, D., *Phys. Rev.*, 1926, v6, 1.
7. Knoll, P., *Sitzungsb. Akad. Wiss. Wien, math.-naturw.*, Cl 1889, v98, 456.
8. Kisch, B., *Exp. Med. and Surg.*, 1951, v9, 333.

Received January 7, 1952. P.S.E.B.M., 1952, v79.

Cyclophorase System XXIV. Oxidation of Pyruvate to Acetate in Rabbit Heart Muscle.* (19376)

M. Fuld[†] and Milton H. Paul.[‡] (Introduced by P. W. Wilson.)

From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisc.

In the cyclophorase-mitochondrial (CM) system pyruvate is oxidized to carbon dioxide and water by way of the citric acid cycle(1). The initial process is the oxidative decarboxylation of pyruvate to a C_2 fragment which in the form of acetyl CoA enters the citric acid cycle by way of condensation with oxalacetate(2). In a recent review(3), it has been stated that homogenates such as those of heart muscle do not remove pyruvate aerobically, unless a dicarboxylic acid is present. This statement is based on the early studies of Ochoa(4), in which it was shown that pyruvate is not oxidized when incubated with dialyzed preparations of minced cat heart muscle.

In view of the fact that a pyruvic oxidase isolated from pigeon breast muscle or pig heart can convert pyruvate quantitatively to acetate and CO_2(5), it seemed logical to expect that the direct oxidation of pyruvate to acetate should also be demonstrable in CM preparations or in homogenates of rabbit heart muscle.

Experimental. The pertinent experimental details have already been covered in the experimental sections of the preceding two communications(6,7).

The estimation of pyruvate and acetate was carried out by the methods respectively of Friedemann and Haugen(8), and of Black(9).

Results. While studying the oxidation of pyruvate in the CM preparations of rabbit heart muscle, we observed that in the presence of malonate, one atom of oxygen was absorbed for each molecule of pyruvate metabolized. In the absence of malonate, approximately 5 atoms of oxygen were absorbed per molecule of pyruvate metabolized (Table I). Thus, depending upon the presence or absence of malonate, the following reactions may be proposed:

TABLE I.
Effect of Malonate on Pyruvate Oxidation.

Malonate present, μM	Oxygen utilized, μatoms	Pyruvate utilized, μM	O/pyruvate
0	71.4	14.2	5/1
15	15.5	13.4	1/1

Complete system consisted of 1 ml of enzyme preparation, .2 ml of .1 M $MgCl_2$, .2 ml of .1 M phosphate buffer, pH 7.4, .3 ml of .01 M adenosine-5-phosphate, 20 μM of lithium pyruvate, .2 ml of 6N KOH in center well with filter paper strip. Vessels gassed with 100% O_2 for 2 min, and equilibrated at 38°C for 5 min before taps closed.

TABLE II.
Effect of Malonate on Pyruvate Metabolism.

Malonate present, μM	Oxygen utilized, μatoms	Pyruvate utilized, μM	Acetate formed, μM	CO_2 formed, μM
50	17.6	18.1	18	17.2

Complete system: see Table I.

$$(1)\ CH_3COCOOH + 0 \longrightarrow CH_3COOH + CO_2,$$
$$(2)\ CH_3COCOOH + 50 \longrightarrow 3CO_2 + 2H_2O.$$

In Table II, additional data from a typical experiment are provided to establish that the requirements of equation (1) are satisfied when pyruvate is oxidized in presence of malonate. The mechanism by which malonate prevents the condensation of the C_2 fragment with oxalacetate is not as yet decided. Either the formation of oxalacetate from pyruvate, or the condensative reaction itself might be the site of action of malonate.

In an attempt to reduce to a minimum any oxalacetate which might arise from endogenous substrate, the enzyme preparation was subjected to dialysis (150 minutes) at 3-5°C against 100 volumes of 0.12 M potassium chloride—0.007 M magnesium chloride (changed 5 times). Under these conditions

---

* This investigation was aided by a grant from the Heart Institute of the National Institutes of Health.

[†] Present address: Graduate School of Public Health, University of Pittsburgh.

[‡] Present address: Army Medical Center, Washington, D.C.

TABLE III. Pyruvate Metabolism After Dialysis of Enzyme Preparation.

Substrate	Oxygen utilized, μatoms	Pyruvate utilized, μM	Acetate formed, μM	CO_2 formed, μM
20 μM pyruvate	19.5	20.1	20.2	17.6
'' + 5 μM fumarate	72.3*			

Complete system: see Table I.
* Fumarate blank subtracted.

the CM preparation, even in absence of malonate, converts pyruvate to acetate (Table III).

A comparable situation exists in the CM system of rat liver where it has been shown by Lehninger(10) that pyruvate is either oxidized directly to CO_2 and H_2O, or in absence of condensing partner (*e.g.* in presence of malonate), is converted quantitatively to acetoacetate. The C_2 fragment, if it is not condensed with oxalacetate, can condense with another C_2 fragment to form acetoacetate. This multiple pathway for pyruvate in liver has been studied extensively by Recknagel and Potter(11).

Discussion. The oxidation of pyruvate by the pyruvic oxidase of animal tissues has been shown by Jagannathan and Schweet(12) to lead to the stage of acetyl CoA, since the product is capable of acetylating sulfanilamide. Acetyl CoA can either condense with oxalacetate to form citrate, as has been shown by Ochoa and Stern(2), or it can be decomposed into acetate and CoA, as shown by Gergely and Hele(13). The latter deacylation reaction is so much slower than the former, that it plays a negligible role except when the condensative reaction is blocked.

Summary. Pyruvate is oxidized to CO_2 and H_2O by the cyclophorase-mitochondrial system of rabbit heart. Under appropriate conditions, *e.g.* by blocking with malonate or by prolonged dialysis of the preparation, pyruvate is oxidized to acetate and carbon dioxide, and the C_2 unit formed does not enter the citric acid cycle by condensation with oxalacetate.

We wish to thank Dr. D. E. Green for his guidance and helpful suggestions.

1. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, 1948, v172, 389.
2. Stern, J. R., and Ochoa, S., *J. Biol. Chem.*, 1951, v191, 161.
3. Ochoa, S., *Physiol. Rev.*, 1951, v31, 56.
4. Ochoa, S., *J. Biol. Chem.*, 1944, v155, 87.
5. Schweet, R., in a "Symposium on Phosphorous Metabolism," Vol. I, Johns Hopkins University Press, 1951.
6,7. Two preceding papers, XXII and XXIII of this series, PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 349, 352.
8. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, 1943, v147, 415.
9. Black, S., *Arch. Biochem.*, 1947, v23, 347.
10. Lehninger, A., *J. Biol. Chem.*, 1946, v164, 291.
11. Recknagel, R. O., and Potter, V. R., *J. Biol. Chem.*, 1951, v191, 263.
12. Jagannathan, V., and Schweet, R. S., *Abst. Am. Chem. Soc.*, Sept., Chicago, 1950, v109, 50c.
13. Gergely, J., and Hele, P., in preparation.

Received January 7, 1952. P.S.E.B.M., 1952, v79.

The Origin of Bradykinin. (19377)

C. G. VAN ARMAN.* (Introduced by W. E. Hambourger.)

From the Rheumatic Fever Research Institute, Northwestern University Medical School, Chicago, Ill.

Bradykinin is a hypotensive and smooth-muscle stimulating substance which is formed from the globulin fraction of plasma when acted upon by proteolytic enzymes such as fibrinolysin, trypsin or snake venom. This substance, discovered by Rocha e Silva, Beraldo, and Rosenfeld(1), is characterized as follows: a) it causes a slow contraction of guinea pig, rat, and rabbit intestine which is not prevented by antihistaminics or atropine,

---

* Present address: G. D. Searle & Co., Chicago, Ill.

b) intravenous injection causes a fall in blood pressure in laboratory animals, c) it is dialysable, stable to heat in acid or neutral solution, and is destroyed by prolonged incubation with trypsin or snake venom, and d) the isolated intestine shows no desensitization after repeated doses. These properties serve to distinguish bradykinin from other gut-contracting substances which may occur in plasma, such as histamine. Bradykinin has been found in the blood of dogs in anaphylactic and peptone shock, but its role is uncertain. In only one of Beraldo's experiments(2) was the amount found sufficient to have been entirely responsible for the fall in blood pressure. The precursor of bradykinin exists only in that portion of the plasma proteins termed the pseudoglobulins, which is precipitated between 35 and 50% saturation with ammonium sulfate(1). According to Neurath *et al.*(3), this includes approximately 21% of the human serum proteins, which when identified by their electrophoretic mobilities are found to be divided as follows, per 100 ml of serum: 0.15 g of albumin, and of the globulins, α_1, 0.12 g; α_2, 0.55 g; β and γ combined, 0.67 g.

It was of interest to determine which of these components contains the precursor. An opportunity to attempt this was afforded by the methods developed for fractionation of plasma proteins by E. J. Cohn and his group at Harvard during recent years. These methods allow sharper separation of the several types of proteins in the native condition than is possible by "salting-out" procedures. Cohn's method No. 10(4) separates the proteins into 6 fractions:

- I —Fibrinogen and anti-hemophilic globulin
- II + III—γ-globulins (antibodies)
  - β_1-globulin, including lipoprotein (complement, midpiece, prothrombin)
  - β_2-globulin (isoagglutinins)
  - plasminogen (profibrinolysin)
- IV —α_1-lipoproteins (carry cholesterol, phospholipids, some steroids)
  - α_2-glycoproteins } (hypertensinogen, cholinesterase, also carries bilirubin)
  - α_2-mucoproteins }
  - β_1-metal combining protein (thyrotropic hormone, iodoprotein)
  - phosphatase
- V —Serum albumin
- VI —traces of IV and V
  - α_1-small acid protein
  - α_2-protein
  - β_1-protein
  - other small proteins and peptides
  - uric acid

These fractions may be further subdivided. The plan of the present experiments was to test each of the 6 main fractions for yield of bradykinin, and then to identify the precursor, so far as possible, by testing the various subfractions.

Methods. Bradykinin was made in 5 batches by treatment of 1-liter portions of bovine pseudoglobulin solutions with snake venom as described by Rocha e Silva *et al.*(1). Potencies measured on the isolated ileum varied considerably. The best preparations were further purified according to the advice of Dr. W. T. Beraldo as follows: Some 600 mg of crude material obtained from 1 liter of bovine plasma were extracted by stirring 3 times with 5 cc portions of glacial acetic acid, and the residue was discarded. Eight volumes of ether were added to the acetic acid solution, and the precipitate was centrifuged down, washed twice with ether, twice with acetone, and dried at 40°C. This material apparently lost no potency during storage in the desiccator for 2 months. Assays were performed with the usual isolated guinea pig ileum in oxygenated Tyrode's solution, atropinized and usually treated with diphenhydramine. Whenever snake venom was used, the ileum was previously densensitized to this agent by repeated doses until no response was obtained.

Experiments and results. The several bovine fractions, separated according to Cohn's method 5(5) were kindly supplied by Dr. James B. Lesh of Armour Laboratories, Chicago, Ill. Method 5 is essentially the same as 10, but the main difference, so far as this study is concerned, would seem to be that Fraction IV is obtained in a more impure state by the older method. Suspensions of each fraction were made in Tyrode's solution at pH 7.6 and centrifuged. One cc of the supernatant solution was incubated at 37°C for 90 seconds with 60 μg (0.06 cc) of a solution of powdered venom from Bothrops jararaca,[†] and 0.1 cc portions were tested at once on the isolated ileum. Fractions I, II + III, V, and VI in 5% suspension yielded no bradykinin, or any other smooth muscle stimulating substance, even upon longer periods of incubation (Fractions III-0 and III-1 yielded a relatively insignificant amount from 5% suspensions). Practically the entire activity of the plasma was contained in Fraction IV, tested as a 1% suspension. The 2 subfractions of IV, termed IV-1 and IV-4, were both very active in 1% suspension. IV-4 yielded approximately twice as much bradykinin as IV-1 (both subfractions had been made from the same lot of plasma). The insoluble part of these fractions did not contain any precursor.

Since narrower subfractions of IV were not available, an attempt was made by other means to discover which component of IV contained the bradykinin precursor. The plan was to find which of the several electrophoretic components would be destroyed or reduced in amount by incubation with snake venom. A 2% suspension was centrifuged at slow speed and the sediment discarded. The solution was dialyzed against distilled water at 2°C for 72 hours and against barbital buffer of pH 8.54 for 24 hours. It was then centrifuged at 19,000 rpm for 45 minutes. A creamy fat layer rose to the top. The solution underneath was still murky but was used nevertheless. It was subjected to electrophoresis‡ in a Tiselius apparatus with a Philpot-Svensson schlieren optical system, at 0.4°C for 128 minutes in barbital buffer of pH 8.54, conductivity 0.00312 $\text{ohm}^{-1}\text{cm}^{-1}$, and ionic strength 0.1, in a field of 6.50 volts/cm. Three peaks were found, corresponding in mobilities to albumin, α- and β-globulins. The relative amounts were estimated by magnification of the photographic curves, with planimetric measurement of the areas under the 3 peaks, which were found to constitute 35, 33, and 32% of the total area, in that order. Sixteen cc of a similar solution of IV, centrifuged at high speed, were then treated with 1.6 mg of snake venom at 42°C for 2 minutes and rapidly cooled, with the object of destroying the bradykinin precursor. After dialysis for 4 days at 2°C as above and further high speed centrifugation, the solution was submitted to electrophoresis under the same conditions. The resulting photographs revealed that the albumin, α- and β-globulins constituted 39, 35, and 25% of the total. This result is not taken to be significantly different from the control, in view of the errors inherent in the technic. There is a slight indication, however, that the β component was reduced more than the others.

Discussion. Fraction IV in Cohn's method 10 comprises approximately 9% of the human plasma proteins(4). It is subdivided into "extract IV-6 + 7", which accounts for 6%, and IV-1, which accounts for 3% of the plasma proteins. It is to be emphasized that bovine and human plasma are different, and with identical treatment yield fractions of somewhat different composition. Using method 5(5), Lesh found that of a final yield of 56 g of dry protein obtained per liter of bovine plasma, Fraction IV-1 constituted 5 g, or 9%, while IV-4 constituted 3 g, or 5%. When made from human plasma, IV-1 contains mostly α-globulin, much of it a lipoprotein containing about 35% lipid, some β-globulin and albumin(6). IV-4 contains nearly lipid-free α- and β-globulins, with some albumin. It contains serum esterase and a specific iron-binding globulin.

Hypertensinogen is present in high concentration in the supernatant after precipitation of Fraction IV-1; however, it is largely inactivated upon precipitation of IV-4 from solution. IV-4 may be further fractionated into IV-5, 6 and 7; IV-6 contains the hypertensinogen(6). The possible physiological reciprocal relationship between hypertensin and bradykinin has been proposed by Rocha e Silva(7). It is of some interest that these two opposing factors, one hypertensive, the other hypotensive, have their origin in a common fraction of the plasma proteins.

The precursor is probably not in the albumin contaminants of IV-4, since Fraction V (albumin) gave no response (this agrees with observations of Rocha e Silva *et al.*). Since

---

† Kindly furnished by Dr. W. T. Beraldo. Original source: Butantan Institute, Sao Paulo, Brazil.

‡ In this work I was fortunate to have the advice and guidance of Dr. Eugene L. Hess.

IV-4 gives a better yield than IV-1, and since the α-globulins are present in lesser amounts in IV-4 than in IV-1, one may suspect that the α-globulins are not responsible; contrariwise, the β_1-globulins are present in greater amount in IV-4, and could be suspected of containing the precursor. The electrophoretic experiment above, although not decisive, points to the β-globulins.

Summary. 1. The precursor of bradykinin is contained in Fraction IV of the bovine plasma proteins (Cohn's nomenclature). 2. Of the 2 main subfractions, IV-4 yields approximately twice as much bradykinin as IV-1.

I wish to thank Dr. C. A. Dragstedt for advice in preparation of the manuscript.

---

1. Rocha e Silva, M., Beraldo, W. T., and Rosenfeld, G., *Am. J. Physiol.*, 1949, v156, 261.

2. Beraldo, W. T., *Am. J. Physiol.*, 1950, v163, 283.

3. Neurath, H., Volkin, E., Erickson, J. O., Craig, H. W., Putnam, F. W., and Cooper, G. R., *Am. J. Syphilis, Gonorrhea and Venereal Dis.*, 1947, v31, 347.

4. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Uroma, E., *J. Am. Chem. Soc.*, 1950, v72, 465.

5. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

6. Edsall, J. T., *Advances in Protein Chemistry*, 1947, v3, 383.

7. Rocha e Silva, M., *Bradicinina*. Thesis, Faculty of Medicine of the University of Brazil, Sao Paulo, 1951.

Received January 9, 1952. P.S.E.B.M., 1952, v79.

Cross-Resistance Studies with Streptomycin, Streptothricin, Neomycin, and Streptolin. (19378)

Joseph F. Pagano, Marvin J. Weinstein, and Richard Donovick.

From the Squibb Institute for Medical Research, New Brunswick, N. J.

Several studies have been reported concerning the cross-resistance of various bacterial species with streptomycin and streptothricin (1-4) and neomycin(5-8). In the present study, cross-resistance has been found among this chemically and biologically related group of antibiotics, but not with every organism studied.

Methods. Strains of *Escherichia coli* (D-56), *Streptococcus fecalis* (ATCC-9790), *Pseudomonas aeruginosa* (D-163), *Micrococcus pyogenes var. aureus* (209P), *Salmonella schottmulleri* (D-51), *Bodenheimer's bacillus* (D-78) and one strain of *Escherichia coli* previously made resistant to chloromycetin, aureomycin, and terramycin, designated *E. coli-R* (D-56-9), were used in this study. Several of these organisms were made resistant to either streptomycin hydrochloride (Squibb, 808 u/mg), streptothricin hydrochloride (Merck, 500 u/mg) or neomycin sulfate (Squibb, 175 u/mg) by repeated exposure to increasing amounts of the antibiotic in yeast beef broth medium (Difco, pH 6.8). Cultures were incubated at 37°C for 1-3 days. The minimal inhibiting concentrations (MIC) of antibiotic were determined by the agar dilution method, the organism being streaked on a series of yeast beef agar plates containing increasing concentrations of the antibiotic. Prior to streaking, the cultures were centrifuged for 30 minutes at 2000 RPM, washed and resuspended in saline. It was noted that the MIC in broth cultures was usually lower than the MIC observed on agar plates. To determine the degree of resistance and cross-resistance developed, an organism made resistant to one of the antibiotics was simultaneously streaked on agar plates containing the other antibiotics. Two-fold increases in concentration of antibiotic were utilized. When the organism grew well at one level and was inhibited completely by the next higher level of antibiotic, the MIC was considered as the mid-point between the two levels. Repeat testing of cross-resistance indicates that the variation

TABLE I. Sensitivity of Parent Culture to Various Antibiotics.

Organism	Strepto-mycin·HCl, Squibb, MIC (mg/ml)	Streptothri-cin·HCl, Merck, MIC (mg/ml)	Neomycin·SO_4, Squibb, MIC (mg/ml)	Strepto-lin, MIC (mg/ml)
E. coli	.0074	.012	.043	.09
S. fecalis	.046	.15	.17	.72
M. pyogenes var. aureus	.0025	.0044	.014	<.03
P. aeruginosa	.015	.02	.057	.24
S. schottmulleri	.0074	.009	.029	.06
Bodenheimer's bacillus	>1.2	.0044	.029	—
E. coli-R	.011	.024	.029	—

TABLE II. Cross-Resistance Behavior.

Organism	Factor* of induced resistance to:	Factor of increased resistance to: Streptomycin HCl (Squibb)	Streptothricin HCl (Merck)	Neomycin HCl (Squibb)
	A. Streptomycin HCl (Squibb)			
E. coli	>338		25	11
	30		33	11
S. fecalis	> 54		1	1
M. pyogenes var. aureus	>480		4	2
P. aeruginosa	>167		10	25
S. schott.	>338		1	2
	19		1	2
E. coli-R	>227		1	2
	B. Streptothricin HCl (Merck)			
E. coli	>250	62		16
S. fecalis	10	1		2
M. pyogenes var. aureus	114	37		24
	34	12		4
P. aeruginosa	38	31		12
Bodenheimer's bacillus	>682	1		12
E. coli-R	>125	42		12
	C. Neomycin SO_4 (Squibb)			
P. aeruginosa	90	8	5	
	16	6	8	
S. fecalis	27	3	2	

$$* \text{ Factor} = \frac{\text{MIC (mg/ml) of resistant strain}}{\text{MIC (mg/ml) of parent strain}}.$$

in resistance observed by this assay method was never greater than 2-fold. An increase in resistance of 4-fold or more is therefore considered significant. The MIC of the parent sensitive strains are shown in Table I. The experimental results obtained are presented in Tables II and III. A number of the resistant organisms were also tested for cross-resistance with different preparations of neomycin [Neomycin sulfate, Pfizer (180 u/mg); Neomycin sulfate, Abbott (250 u/mg); Neomycin B hydrochloride, Squibb (280 u/mg); Neomycin C hydrochloride, Squibb (170 u/mg); a Squibb preparation of streptothricin sulfate (161 u/mg); and the antibiotic streptolin hydrochloride (30 u/mg)]. The results obtained are presented in Table III. With all organisms, attempts were made to induce the initial resistance to each of the individual antibiotics before testing for cross-resistance, however, where such strains are not reported, difficulty was encountered in inducing resistance.

Results. E. coli. Resistance to streptomycin or streptothricin resulted in cross-resistance (Table II). When the resistance to streptomycin was increased from 30-fold to greater than 338-fold, there was no detectable increase in the level of cross-resistance.

S. fecalis. An increase in resistance to either

TABLE III. Cross-Resistance Behavior to Streptolin and Different Preparations of Neomycin and Streptothricin.

			Factor of increased resistance to:					
Organism	Antibiotic* and factor† of increase in resistance		Neomycin SO_4 (Pfizer)	Neomycin SO_4 (Abbott)	Neomycin B HCl (Squibb)	Neomycin C HCl (Squibb)	Streptothricin SO_4 (Squibb)	Streptolin HCl
E. coli	Stm	>338	10	6	6	5	40	8
	Str	>250	6	5	5	5	>129	32
S. fecalis	Stm	> 54	1	1	1	1	1	1
	Str	10	1	2	2	1	3	4
M. pyogenes var. aureus	Str	114	20	19	18	10	> 59	>24
P. aeruginosa	Stm	>167	9	10	8	4	10	4
	Str	38	10	8	>6	3	6	16
	Neo	90	>36	36	17	>4	2	—
S. schott.	Stm	>338	1	2	1	1	> 1.5	4

* Stm—Streptomycin·HCl (Squibb); Str—Streptothricin·HCl (Merck); Neo—Neomycin·SO_4 (Squibb).

$$\dagger \text{ Factor} = \frac{\text{MIC (mg/ml) of resistant strain}}{\text{MIC (mg/ml) of parent strain}}.$$

streptomycin, streptothricin, or neomycin was not accompanied by cross-resistance.

Micrococcus pyogenes var. aureus. Resistance to streptothricin resulted in a simultaneous increase in resistance to streptomycin and neomycin. Further increases in resistance to streptothricin resulted in an increase in the level of cross-resistance which was approximately proportional to the increase in streptothricin resistance. A greater than 480-fold increase in streptomycin resistance, however, did not result in any cross-resistance to neomycin or any significant cross-resistance to streptothricin.

P. aeruginosa. Resistance to streptomycin or streptothricin resulted in cross-resistance. When made resistant to neomycin (90-fold), only a 5 to 8-fold increase in resistance to streptomycin or streptothricin was observed. Increasing the level of neomycin resistance from 16-fold to 90-fold did not result in any increase in cross-resistance.

S. schottmulleri. A greater than 338-fold increase in resistance to streptomycin resulted in no increase in resistance to streptothricin or neomycin.

Bodenheimer's bacillus. A greater than 682-fold increase in resistance to streptothricin resulted in a 12-fold increase in resistance to neomycin.

E. coli-R. After being made resistant to streptothricin, a simultaneous increase in resistance to streptomycin and neomycin was observed. However streptomycin resistance did not result in cross-resistance to streptothricin or neomycin.

Cross-resistance with streptolin and various preparations of neomycin and streptothricin (Table III). *E. coli* resistant to streptomycin or streptothricin, *Micrococcus pyogenes var. aureus* resistant to streptothricin, and *P. aeruginosa* resistant to streptothricin, all showed cross-resistance with streptolin as well as with the other antibiotics used in this study. *S. fecalis* made resistant to streptomycin showed no cross-resistance and when made streptothricin-resistant showed minimal (4-fold) resistance to streptolin. Streptomycin-resistant *S. schottmulleri* showed a minimal (4-fold) increased resistance to streptolin. In no case do the latter two organisms show cross-resistance with any of the other antibiotics used in this study.

When the organisms shown in Table III were tested for cross-resistance to the different preparations of neomycin and a Squibb preparation of streptothricin, cross-resistance was confirmed in every case. Resistance levels are of the same order of magnitude and the slight variations in MIC levels are not considered significant.

Discussion. In the present study, and confirming previous reports with other strains of *E. coli*(5,7,9,10), *E. coli-R* (D56-9) made resistant to streptomycin showed no cross-resistance with streptothricin and neomycin. However, *E. coli* (D-56), resistant to streptomycin showed a simultaneous increase in resistance to streptothricin and neomycin. It is evident that within the same species there exists the potentiality for developing strains which may or may not show cross-resistance. The mechanism governing this phenomenon is now under investigation.

The strain of *S. fecalis* used in these studies appears to develop mono-resistance. Resistance to either streptomycin, streptothricin, or neomycin was not accompanied by a simultaneous increase in resistance to the other antibiotics considered in this investigation. Since a limited number of resistant cultures were examined, no extensive generalizations can be made concerning the observed lack of cross-resistance in this organism. Further study may reveal in *S. fecalis* the two types observed in strains of *E. coli.*

No cross-resistance with neomycin was observed when *M. pyogenes var. aureus* or *E. coli-R* was made initially resistant to streptomycin. The former organism showed only a minimal (4-fold) increase in resistance to streptothricin while the latter showed no resistance. However, when made initially resistant to streptothricin, both organisms showed cross-resistance to streptomycin and neomycin. It would appear that cross-resistance in these two organisms is related to the pathway of development of the initial resistance. In order to elucidate this behavior a more detailed study of the various types is necessary. The cross-resistance behavior observed provides evidence for considering streptomycin, streptothricin, neomycin, and streptolin as a biologically as well as a chemically related group of antibiotics. When a given organism was made resistant to one antibiotic, its cross-resistant behavior with the other 3 antibiotics was consistent. The organism either showed increased resistance to all of the other 3 antibiotics or to none of them. A biological relationship based on cross-resistance patterns is now well established for the penicillins (11, 12), streptomycins(13,14) and between aureomycin, chloromycetin, and terramycin(15-18).

In the present studies, the inhibiting concentration of an antibiotic for an organism was obtained by the agar dilution method. This procedure permits distinguishing total population growth from that of individual colonies appearing along the streak line. Such colonies may be mutants which arise in the population and do not represent the resistance level of the total population. Determination of culture sensitivity to an antibiotic in a liquid medium does not permit visual differentiation between the resistance of the total population and that of the individual cells.

Summary. Cross-resistance of a number of organisms to streptomycin, streptothricin, neomycin, and streptolin has been observed by the agar dilution method. Variability of cross-resistance behavior within a species is reported.

---

1. Sullivan, M., Stahly, G. L., Birkeland, J. M., and Myers, W. G., *Science,* 1946, v104, 397.

2. Hayano, S., *Jap. J. Antibiotics,* 1949, v2, 281.

3. Hosoya, S., Solda, M., Komatsu, N., Iwasaki, M., Sonoda, Y., and Uchida, M., *Jap. J. Exp. Med.,* 1949, v20, 117.

4. Kuroya, M., Ishida, N., Katagiri, K., Konno, J., Kikuchi, M., and Mizuguchi, R., *Jap. J. Antibiotics,* 1950, v3, 223.

5. Waksman, S. A., Lechevalier, H. A. and Harris, D. A., *J. Clin. Invest.,* 1949, v28, 934.

6. Waksman, S. A., and Lechevalier, H. A., *Antibiotics and Chemotherapy,* 1951, v1, 125.

7. Demerec, M., and Demerec, R., *Bact. Proc.,* 1950, 101.

8. Gezon, H. M., and Fasan, D. M., *Science,* 1951, v114, 422.

9. Waksman, S. A., and Lechevalier, H. A., *Science,* 1949, v109, 305.

10. Kelner, A., and Morton, H. E., *J. Bact.,* 1947, v53, 695.

11. Eisman, P. C., Marsh, W. S., and Mayer, R. L., *Science,* 1946, v103, 673.

12. Dowling, H. F., Hirsh, H. L., and O'Neil, C. B., *J. Clin. Invest.,* 1946, v25, 665.

13. Rake, G., McKee, C. M., Pansy, F. E., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 107.

14. Donovick, R., and Rake, G., *J. Bact.,* 1947, v53, 205.

15. Herrell, W. E., Heilman, F. R., and Wellman,

W. E., *Ann. N. Y. Acad. Sci.*, 1950, v53, 448.

16. Pansy, F. E., Khan, P., Pagano, J. F., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 618.

17. Kaipainen, W. J., *Ann. Med. Exp. Biol. Fenn.*, 1951, v29, Supple. I.

18. Fusillo, M. H., and Romansky, M. J., *Antibiotics and Chemotherapy*, 1951, v1, 107.

Received January 16, 1952. P.S.E.B.M., 1952, v79.

Blood Changes of Normal Dogs During Chronic Blood Volume Expansion with Dextran.[*] (19379)

L. H. MARSHALL, C. H. HANNA, HEINZ SPECHT, AND P. A. NEAL.

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Md.

Blood changes occurring during long continued administration of dextran have been studied in normal dogs.

Methods. Three litter mate mongrel bitches weighing 13-16 kg were fed equal parts dog chow and horse meat in amounts adjusted to maintain body weight. They received intravenously 10, 15 and 20 ml dextran solution (Macrodex[†]) per kg respectively, 3 times weekly for 13 weeks. This amounted to approximately 300, 500 and 600 g dextran respectively. A control dog from the same litter received 10 ml/kg physiological saline. Preceding the injections 8 ml of blood were withdrawn without stasis and heparinized. Hematocrit values were determined in Wintrobe tubes spun 30 min at 1050 G. Estimations of relative viscosity were made within 20 min of withdrawing the sample in an Ostwald viscosimeter at 3 different pressures. Specific gravities of whole blood and of plasma were measured by the copper sulfate method. Values for the sedimentation of the cells after standing undisturbed for 60 min were obtained within 3 hrs of withdrawing the sample. Hemoglobin was converted to acid hematin and read on the Klett-Summerson photoelectric colorimeter. Mean arterial blood pressures were recorded at weekly intervals by direct puncture of a femoral artery. Estimations of glomerular filtration rate (creatinine), effective renal plasma flow and maximal tubular transfer (p-amino hippurate) were made(1) on 2 dogs receiving dextran and on the control before and after 7 to 12 wks of injections.

Results. The repeated injections of dextran were well tolerated. Body weights, after an initial fall during the control period, were maintained with an increase in food intake of 25 g (10-12.5% of the total diet). Mean arterial blood pressures varied within normal limits. Plasma specific gravity was 1.022 to 1.024 in all dogs throughout the experiment. The renal function tests revealed no significant variations from the control. In agreement with the report of Thorsen(2) no significant pathological changes were found by microscopic examination[‡] of the tissues of the dog that received 10 ml/kg Macrodex. The tissues of this dog were fixed in aqueous formalin and therefore were not suitable for demonstrating possible dextran deposits.

Average weekly changes in the hematocrit percentage and relative viscosity are shown in Fig. 1, where the mean of the preinjection

TABLE I. Preinjection Mean Values Taken as 100% in Fig. 1 for Hematocrit Volume and Relative Viscosity.

Subsequent treatment Sol.	Dose, ml/kg	Hematocrit vol, %	Relative viscosity
Saline	10	41.4	4
Dextran	10	46.9	4.68
	15	48.8	4.88
	20	41.3	4.01

---

[*] Presented before the American Physiological Society, Salt Lake City, Utah, Sept. 6-8, 1951.

[†] Solution used was the Swedish product "Macrodex," 6% dextran in 0.9% NaCl.

[‡] We are indebted to Dr. Benjamin Highman of the Laboratory of Pathology and Pharmacology for this examination.

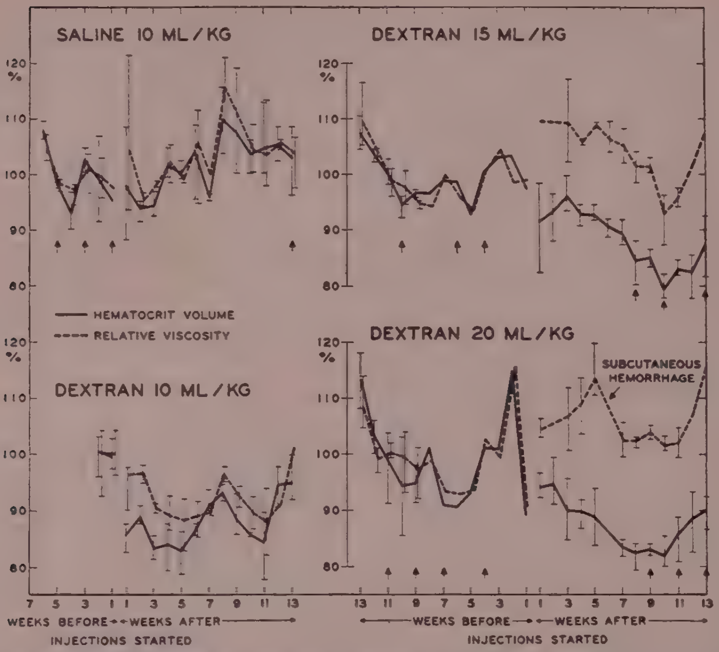


FIG. 1. Comparison of changes in hematocrit volume and relative viscosity in the control dog and in 3 dogs receiving Macrodex in varying amounts. The mean of the preinjection values equals 100%. Each point with bars represents the weekly average of 2 or 3 determinations and their range; absence of bars indicates only 1 determination was made that week. Arrows indicate renal clearance tests. Two additional renal clearances were made on the control dog during the 16th and 19th weeks of injection.

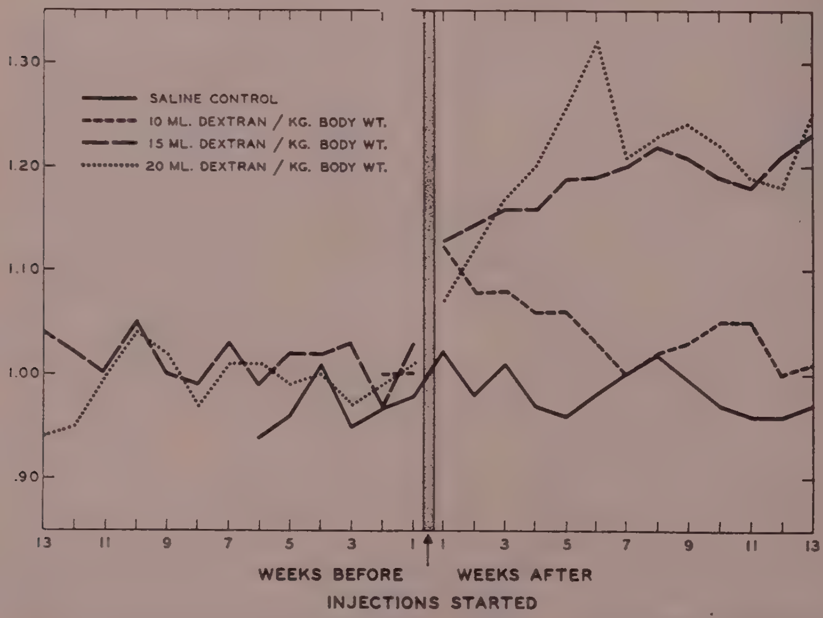


FIG. 2. Ratio of values shown in Fig. 1 for viscosity (× 10) and hematocrit volumes.

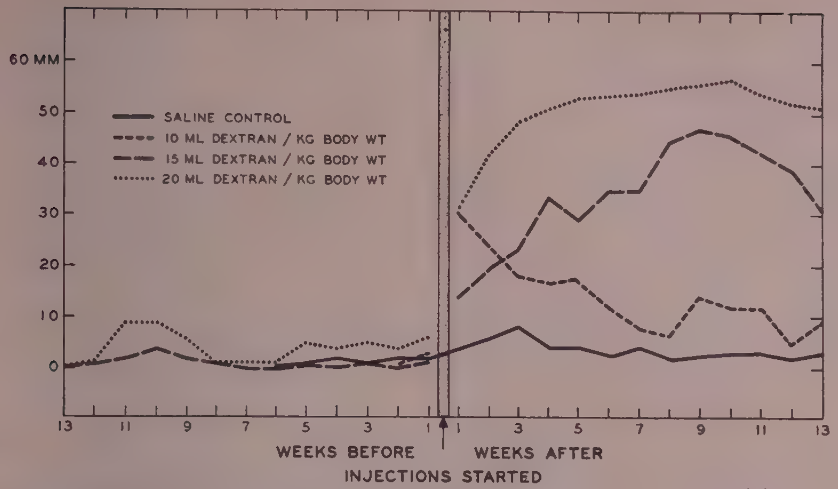


FIG. 3. Sedimentation rates (uncorrected weekly averages) in mm/hr of dogs receiving dextran and the control receiving saline.

values equals 100% (Table I). The extremely high values found in the 20 ml/kg dog for the single blood sample taken the second week before injections began were due to inadvertent dehydration. To facilitate comparison between dogs, use has been made of the ratio of these changes (Fig. 2). The mean weekly sedimentation rates uncorrected for red cell content are shown in Fig. 3. Specific gravity of whole blood and the hemogloblin values followed the hematocrit readings, so there was no change in the ratio of hemoglobin to mean cell volume.

Discussion. Whether or not the initial decrease in hematocrit percentage after starting the injections was due wholly to dilution is not indicated satisfactorily by the evidence presented. If dilution were the sole factor, the smallest dosage should have produced the least change in the ratio of cells to plasma. Actually, the largest initial decrease was with this dosage, while the smallest initial change occurred in the dog receiving most dextran. Hematocrit decreases attained subsequent to the initial values were consistent with the dosage administered. Whittaker and Winton (3) have shown the depressing effect upon blood viscosity of a reduction in hematocrit value. In spite of this decrease the relative viscosity of the blood of the 2 dogs receiving the largest injections was increased. This would indicate that injected dextran solution, by virtue of a relative viscosity approaching that of whole blood (Macrodex is slightly more than 3 times more viscous than water; plasma relative viscosity is 1.5) (3), compensates in part for the loss or dilution of erythrocytes while expanding the circulating volume.

The greatest increase in sedimentation rate with the largest injections of dextran confirms previous reports(4). Since the rates are uncorrected for numbers of erythrocytes, these changes are partially due to the hemodilution. To evaluate the importance of this factor, normal dog bloods were diluted with their own plasmas or with Macrodex to hematocrit readings approximating 90, 80 or 70% of the original value. Sedimentation rates in the plasma-diluted bloods averaged 67 to 74% of the rates found in the bloods diluted with Macrodex. It has been shown (5) that the tendency to produce erythrocyte aggregation and hence increase sedimentation is directly dependent on the molecular size of dextran above 60,000. The diminished sedimentation reaction as the injections continued in the dog receiving 10 ml/kg may signify an enhanced capacity to form smaller molecules from the freshly injected material. Evidence of a diminished reaction to repeated injections has been reported in rats(6).

The gradual disappearance of dextran from the blood stream after injection is generally accepted as not being due to quantitative storage. The recent report(7) of increased glycosuria in phlorizinized starved dogs after dextran administration indicates that metabolism to glucose is one avenue of disposal. There is evidence in mice of transfer to exhaled CO_2(8). Partial degradation to smaller molecules capable of passing the renal glomerulus constitutes another possibility.

Summary. Except for a decrease in hematocrit volume, intravenous administration of dextran continued for 13 weeks produced in 3 normal, nonshocked dogs no demonstrable harmful effects. Blood viscosity was maintained at or above control levels when the injections were 15 to 20 ml/kg body weight. The dog receiving 10 ml/kg exhibited a tendency to adjust to the foreign polysaccharide as evidenced by a return towards normal hematocrit values and sedimentation rates in spite of continued dextran.

---

1. Marshall, L. H. and Specht, Heinz. In preparation.

2. Thorsen, G., *Lancet,* 1949, v256, 132.

3. Whittaker, S. R. F. and Winton, F. R., *J. Physiol.,* 1933, v78, 339.

4. Bull, J. P. *et al., Lancet,* 1949, v256, 134.

5. Hardwicke, J., Ricketts, C. R., Squire, J. R., *Nature,* 1950, v166, 988.

6. Vorhees, A. B., *et al.,* Proc. Soc. Exp. Biol. and Med., 1951, v76, 254.

7. Gray, Irving, Siiteri, P. K., Pulaski, E. J., Proc. Soc. Exp. Biol. and Med., 1951, v77, 626.

8. Cargill, W. H. and Bruner, H. D., Abstract, *Am. Soc. Pharm. Exp. Ther.,* Fall Meeting, 1951.

Received January 17, 1952. P.S.E.B.M., 1952, v79.

Sarcomas Induced in Rodents by Imbedding Various Plastic Films.* (19380)

B. S. Oppenheimer, Enid T. Oppenheimer, and Arthur Purdy Stout.
From The Cancer Research Institute, College of Physicians and Surgeons, Columbia University, N.Y.

In 1948 we reported that sarcomas of various types could be induced in albino rats in a significant percentage of cases by imbedding cellophane film subcutaneously in the anterior abdominal wall, or by wrapping it around one kidney(1). Since then the experiments have been extended by imbedding cellophane film in albino and black mice, by testing alcohol-extracted cellophane, and by employing various other plastic films unrelated chemically to cellophane. In addition several other materials were imbedded in an effort to learn the nature of the carcinogenic agent or mechanism involved in the procedure. The results of these new experiments are herewith reported.

Method. The method previously described (1) was used, except that the film or other material was always inserted in the anterior abdominal wall, and no further experiments were attempted with wrapping the film around the kidney. The experiments were conducted over a period of 4 years. Some overlapped. The food cages, handling and room temperature were essentially the same. The rats were all albinos, of the Wistar strain. Films were usually sterilized by immersion in 80% alcohol and then washed in sterile saline solution before imbedding. Occasionally sterilization of the plastic film was accomplished by autoclaving, or by benzalkonium chloride ("Zephiran") 1:1000 or weaker. As malignant tumors arose after any one of these methods of sterilization, it is improbable that the particular technic of sterilization played a role in the result. The malignancy of the tumors produced was established by histologic examination and usually also by transplantability, as previously described(1). Experiments lasted 1-2 years. In calculating the percentage of positive results, *i.e.*, production of tumors, the figures were based on the number of animals that survived the minimum period neces-

---

* This work was supported by a grant from the National Cancer Institute, Public Health Service.

sary for tumor production. In a few instances the imbedded film could not be found at post-mortem examination, probably because it had ulcerated out; such cases were not included in the totals. The imbedded plastic film was almost invariably enveloped by a thin membranous sac from which it could be readily removed. This sac was often found one week after imbedding the film. The sarcoma, if present, was adherent to the outer surface of this sac, and not to the film itself.

Results with cellophane film imbedded in mice. The cellophane film was regenerated cellulose seamless tubing of .0039″ thickness, as in previous experiments on rats(1). Squares of cellophane film, 1 cm in diameter, were imbedded in 50 albino mice of the Longacre strain. The results were as follows:

Shortest time of appearance of a malignant tumor	245 days
No. of mice surviving over 244 days	35
No. of malignant tumors	8
Tumors	22.8%

The tumors consisted of 7 fibrosarcomas and one rhabdomyosarcoma. All the tumors except one were transplantable, sometimes to the 4th tumor generation.

Cellophane imbedded in black mice (C57) yielded only one tumor in 23 effective imbeddings. This tumor was, however, a typical fibrosarcoma and was transplantable to the 3rd tumor generation. (It is known that this strain of black mice is unusually resistant to artificial tumor production.)

Experiments with linters in rats. Dry wads of cotton fiber, known as linters, from which this cellophane was manufactured, were imbedded in 50 Wistar albino rats. The linters were sterilized by autoclaving. No tumors resulted. In all instances in which histologic examination was carried out, a foreign-body reaction, never a sarcoma, was found. These experiments show that the carcinogenic agent was probably not the original linters from which the cellophane was manufactured.

Dry wads of sterile surgical cotton were inserted into 5 albino rats. After 468 to 634 days, no tumors resulted; only foreign-body reactions around the cotton fibers were found.

Results with alcohol-extracted cellophane in rats. As the nature of the carcinogen in the original cellophane was unknown, an endeavor was made to remove impurities by extracting the film as thoroughly as possible with hot methanol for 24 hours in a Soxhlet apparatus, kindly done by Dr. Hans T. Clarke. The extract itself when injected subcutaneously into rats, produced no tumors. On the other hand, sarcomas were produced in a high percentage of rats by imbedding the extracted film (called by us "cellophane B".) Results of imbedding alcohol-extracted cellophane ("cellophane B"):

No. of rats used		50
Shortest time of appearance of tumor		263 days
No. of rats surviving over 260 days		44
Total No. of malignant tumors		20
Fibrosarcomas	12	
Incipient sarcomas	3	
Liposarcomas	1	
Osteogenic sarcomas	2	
Myxoma (malignant)	2	
Tumors		45.4%

On removal from the rat the alcohol-extracted cellophane film usually showed opaque patches and was less flexible and more brittle than on insertion. In contrast, other plastic films appeared as transparent and almost as glossy after imbedding as on insertion.

Results with polyethylene film in rats and mice. A commercial polyethylene film, 0.002 mm thick, was first tested, as this was being used by surgeons; subsequently a specially prepared pure polyethylene was employed. Results with the commercial polyethylene were as follows:

No. of rats imbedded		98
Shortest time of appearance of tumor		392 days
No. of rats surviving 392 days		80
No. of malignant tumors		10
Fibrosarcoma	8	
Rhabdomyosarcoma	1	
Osteogenic sarcoma	1	
Tumors		12.5%

Polyethylene is not always a pure product. For example, it may contain dicetyl phosphate which is a violent irritant to tissues. Since it seemed possible that the carcinogenic action might be due to the presence of such additives or impurities, a very pure polyethylene was prepared with no additive of any kind. We have reason to believe that this polyethylene film is as pure as can be manufactured. The film on insertion was glossy and transparent,

TABLE I. Results of Imbedding Plastic Films, etc. in Rodents.

Material imbedded	Animals used	No. imbedded and surviving‡	Malignant tumors produced		Transplants made	No. animals still alive
			No.	%		
Cellophane (A) (original)*	Albino rats	42	15	35.7	To 5th tumor generation	0
”	” mice	35	8	22.8	4th	0
”	Black mice	23	1	4.3	3rd	0
Cellophane (B) after extraction with alcohol	Albino rats	44	20	45.4	2nd	0
Commercial polyethylene	” ”	80	10	12.5	3rd	0
Pure polyethylene	” ”	40	5†	12.5	1st	15
”	” mice	28	3	10.7	1st	1
Vinyl film	” rats	44	14†	31.8	2nd	5
Cotton linters	” ”	50	0	0	—	0
Surgical cotton	” ”	5	0	0	—	0
Glass cover-slips	” ”	50	0†	0	—	23

* The experiments with this original Cellophane (A) appeared in our previous publication, and are included here for completeness.

† Incomplete.

‡ Surviving minimal time for tumor production.

and when removed from the animal after prolonged imbedding, showed little or no change.

Results of imbedding pure polyethylene film in rats and mice. To the present, this film on imbedding in rats produced 12.5% fibrosarcomas. Fifteen rats are still alive, about 20 months after imbedding the film. In another series of experiments with albino mice (Paris strain) 10% of fibrosarcomas were produced. Only one mouse is still alive 19 months after imbedding. It is possible, but improbable, after such long intervals that more sarcomas will appear in these animals.

Results of imbedding vinyl chloride film in rats. This is an opaque film. After imbedding the only obvious change was less flexibility. The results were as follows:

No. of rats imbedded with film		45
Earliest time of appearance of tumor		189 days
No. of rats surviving for over 189 days		44
No. of malignant tumors		14
Fibrosarcoma	12	
Liposarcoma	1	
Unclassified malignant sarcoma	1	
Five rats are still alive, about 22 months after operation.		
Tumors—to date		31.8%

Results of imbedding glass cover-slips in anterior abdominal wall of rats. Since cellophane, polyethylene, and vinyl chloride film all produce sarcomas in a variable percentage, and yet appear to have no common chemical factor to explain their carcinogenic effect, the question of chronic mechanical irritation arose. Therefore chemically-clean round glass cover-slips, approximately 0.2 mm thick and 18 mm in diameter, were imbedded subcutaneously in the anterior abdominal wall of 50 rats. At post-mortem examination these cover-glasses were occasionally found to be cracked or splintered. Up to the present, 12 to 14 months after insertion, no tumors have been produced, but 23 rats are still alive. These negative results, combined with those of experiments with linters and surgical cotton, afford avidence that the chronic irritation alone of these foreign bodies does not produce tumors. They also serve, in a measure, as controls for our positive results with plastic films.

A summary of the results of all our experiments is given in Table I. For the sake of completeness we have included the results of our previously published experiments on the original cellophane (called by us "cellophane A".) Throughout this series, tumors reported as artificially induced all arose at the site of imbedding of the plastic films. Among the 636 autopsies performed on these rodents, 13 spontaneous tumors were found, but these could not be confused with the artificially induced tumors, since they arose at sites other than the place of imbedding.

Discussion. The results of these experiments are of both theoretical interest and possibly of some practical importance. At present we have no explanation of the mechanism by which the imbedding of these particular plastic films results in sarcomas of various types. Histologically these tumors resemble those obtained in rodents by the injection of benzpyrene; but the plastic films used here are all polymers, not aromatic hydrocarbons. One should recall that Turner(2) found accidentally that bakelite disks implanted for long periods in albino rats produced fibrosarcomas. As yet we have not found a plastic film which did not produce sarcomas, but other materials are now being tested with our technic.

The question of mechanical irritation and carcinogenesis is an old and broad one, and is still open. Our evidence does not favor mechanical irritation as the cause by itself of tumor production. In endeavoring to find an explanation for the carcinogenic effect of these films, differing as they do from one another in chemical composition and with no apparent common chemical factor, one obvious suggestion is that the tumor is caused by some impurity, such as an additive or plasticizer used in their manufacture. This explanation is rendered less likely by the finding that cellophane thoroughly extracted with alcohol, whereby most of such impurities would be removed, is at least as carcinogenic as the original film. Moreover, the pure polyethylene, without additives, produces about the same percentage of tumors as the commercial film. As to possible practical implications of these experimental results, it is known that plastics are now being used for a great variety of very useful purposes by surgeons. It must be emphasized again that so far there is no reported instance in which the imbedding of a plastic film in a human being has resulted in a malignant tumor.

Summary. 1. Sarcomas have been produced in rats by imbedding subcutaneously various plastic films, namely cellophane, an alcohol-extracted cellophane, a commercial polyethylene, a very pure polyethylene and vinyl chloride film. 2. Sarcomas have been produced in mice, but in a lesser percentage of cases, by imbedding cellophane and pure polyethylene film. 3. Apparently impurities and additives in the films do not account for the production of these malignant tumors. 4. So far every plastic film tested has produced sarcomas in a certain percentage of instances. 5. Other foreign bodies, such as linters, surgical cotton and glass cover-slips similarly imbedded in the anterior wall of rats have not produced malignant tumors. 6. No adequate explanation of the mechanism of this carcinogenic effect of imbedded plastic films has been found. 7. The experiments have been limited to rats and mice.

Conclusion. A group of agents, all polymers, and differing from previously known carcinogens, has been found to be carcinogenic for rats and mice.

---

1. Oppenheimer, B. S., Oppenheimer, Enid T., and Stout, Arthur Purdy, PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 33.

2. Turner, Floyd C., *J. Nat. Cancer Inst.*, 1941, v2, 81.

Received January 25, 1952. P.S.E.B.M., 1952, v79.

Effects of Vitamin B_{12} and Penicillin on Thiouracil Action in Chicks.* (19381)

DAVID LIBBY AND JOSEPH MEITES.†

From the Departments of Poultry Husbandry, Physiology and Pharmacology, Michigan State College, East Lansing, Mich.

Retardation of growth in young rats fed 0.1% thiouracil(1) or following parathyroidectomy(2) on a vit. B_{12}-deficient diet was at least partially counteracted by vit. B_{12} supplementation. Survival rate in the latter rats was also significantly increased by vit. B_{12}. Although thyroid hyperplasia in some of the thiouracil-treated rats appeared to be prevented by vit. B_{12}, subsequent work in this laboratory and by Greer(3) showed that the vitamin does not modify this action of goitrogens.

Vit. B_{12} and penicillin have both been shown to stimulate growth in chicks. The vitamin is believed to enhance protein synthesis(4,5) and increase nitrogen retention (6), while penicillin has recently been demonstrated to spare vit. B_{12}, folic acid, choline, riboflavin, and pantothenic acid in the chick (7). Since the hypothyroidism accompanying thiouracil administration results in decreased body growth and appetite, it was of interest to see whether vit. B_{12} and penicillin would counteract these effects in chicks. It was also pertinent to determine whether the vitamin and antibiotic would modify the action of thiouracil on thyroid and comb weight.

Procedure. In the first experiment, day old, single comb, male White Leghorn chicks were divided into 8 uniform groups of 12 each, and were fed the vit. B_{12}-deficient ration of Miller and Groschke(8) for one week. For the next 4 weeks, the ration was supplemented with 0.1% thiouracil and/or 0.5 to 4.0 μg % vit. B_{12},‡ according to the grouping shown in Table I. Body weight and food consumption were measured weekly. At the end of 4 weeks, all birds were sacrificed and the thyroids and combs were removed and weighed. In the second experiment, day-old Rhode Island Red chicks were approximately evenly divided between males and females into 6 groups of 12 each. They were initially placed on the vit. B_{12}-deficient diet for 2 weeks, and for the following 3 weeks the diet was supplemented with 0.1% thiouracil, 4.0 μg % vit. B_{12} or 2 mg of procaine penicillin G per pound of food. The optimal level of vit. B_{12} for growth in Rhode Island Red chicks had previously been shown to be about 2.0 μg %(8), and hence the chicks in this experiment were receiving about twice the optimal level. At the end of 3 weeks, the birds were killed and the thyroids were removed and weighed. The chicks in both experiments were maintained on wire floors in battery brooders, with feed and water available at all times.

Results. The data from the first experiment are summarized in Table I. The addition of 0.5 to 4.0 μg % vit. B_{12} (Groups 2, 3, 4) to the basal ration increased body weight and food intake above the controls (Group 1). No explanation can be offered as to why 2.0 μg % vit. B_{12} proved less effective in these respects than 0.5 μg % vit. B_{12}. The vitamin did not alter thyroid or comb weight. Thiouracil (Group 5) markedly reduced average body weight gains and appetite, and more food was required per g of gain in body weight. Thyroid weight was increased about 12-fold while the combs weighed only about ⅛th as much as in the controls. The latter indicates that thiouracil drastically reduced androgen levels in the body. Vit. B_{12} (Groups 6, 7, 8) partially counteracted the depression of growth and appetite by thiouracil, but did not alter its action on thyroid and comb weight. It can be seen that the larger doses of vit. B_{12} were more effective in preventing growth

---

* Published with the approval of the Director of the Michigan Agric. Exp. Station as Journal Article No. 1315.

† These studies have been aided in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

‡ Crystalline vit. B_{12} was kindly supplied by Merck and Co., Rahway, N. J.

TABLE I. Effects of Vit. B_{12} on Thiouracil Action in Male Leghorn Chicks, 8 Groups.

Treatment	Avg body wt, g		Avg food intake		Avg thyroid wt per 100 g body wt, mg	Avg comb wt per 100 g body wt, mg
	Initial	Gain	Daily	Per g gain body wt		
Controls	59.7	270.4	22.3	2.3	8.3 ± .4*	575 ± 19.8*
.5 μg % B_{12}	59.1	324.6	25.7	2.4	8.5 ± .3	655 ± 21.3
2 ”	61.5	290.5	24	2.4	8.7 ± .4	569 ± 16.8
4 ”	58.8	344.5	26.9	2.2	7.6 ± .4	537 ± 22.5
T†	59.6	151.3	13	3.2	99.9 ± 21	67 ± 6.4
T + .5 μg % B_{12}	58.5	161.3	16.6	3.4	89.8 ± 14.5	66.4 ± 7.9
T + 2 ”	59	177.1	17.4	3.3	86.5 ± 10.4	64.2 ± 10.2
T + 4 ”	59	192	20.2	2.9	115.8 ± 19.6	48.2 ± 7.5

* Stand. error of mean $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

† T = Thiouracil.

TABLE II. Effects of Vit. B_{12} and Penicillin on Thiouracil Action in Rhode Island Red Chicks, 6 Groups.

Treatment	Avg body wt, g		Food/gain ratio	Avg thyroid wt/100 g body wt, mg
	Initial	Gain		
Controls	88	125.5	3.27	15.7
4 μg % B_{12}	86	140.1	3.08	17.5
T*	88.7	97.1	4.07	153.4
T + 4 μg % B_{12}	88.5	123.7	3.05	143.4
T + penicillin	76	137.3	2.81	138.4
T + 4 μg % B_{12} + penicillin	75.2	154.2	2.59	159

* Thiouracil.

inhibition than the smaller doses.

In the second experiment (Table II), 4.0 μg % vit. B_{12} (Group 2) enhanced body growth and appetite of the Rhode Island Red chicks, but apparently this vitamin level was not as effective as in the Leghorn chicks. This suggests that the Rhode Island Red chicks had greater initial reserves of vit. B_{12} in their bodies than the Leghorn chicks. Thiouracil (Group 3) reduced average body weight gains and appetite, and increased the food/gain ratio. Vit. B_{12} (Group 4) and penicillin (Group 5) completely overcame the thiouracil-induced inhibition of growth, while the combination of the vitamin and antibiotic was considerably more effective than either alone. Thyroid hyperplasia in the thiouracil-treated chicks was not prevented by the vitamin or antibiotic.

Discussion. In general, these data on chicks are in agreement with those previously reported in rats(1,2). Thiouracil reduced body growth and appetite in both species, and these were at least partially counteracted by vit. B_{12}, or by penicillin in the chicks. It is believed to be significant that the greater doses of vit. B_{12} were more effective than the lower doses in overcoming growth inhibition by thiouracil in the Leghorn chicks. This suggests that still higher levels of vit. B_{12} than used might have resulted in more complete counteraction of growth inhibition. The favorable action of penicillin on growth in the Rhode Island Red chicks cannot be attributed entirely to a sparing of vit. B_{12} since it proved to be more effective than twice the optimal dose of the vitamin. It appears probable that the antibiotic also made larger quantities of other vitamins available to the chicks.

Neither the normal thyroids of the control chicks nor the greatly hyperplastic thyroids of the thiouracil-treated chicks were influenced by vit. B_{12} or penicillin. Similarly, vit. B_{12} or antibiotics failed to alter thyroid activity in euthyroid(9), hypothyroid(1) or hyperthyroid rats(9-11), despite stimulating body growth in these animals. This suggests that the favorable actions of vit. B_{12} and antibiotics on growth are exerted independently of any effect on thyroid function. However, it does not preclude the possibility that marked changes in thyroid activity can alter normal nutritional requirements of the body. Thus hypothyroidism generally decreases body growth and is believed to reduce dietary requirements, while severe hyperthyroidism also reduces body growth but is believed to increase nutritional requirements(12). If one considers normal body growth as a criterion of adequate dietary intake, then the favorable effects of vit. B_{12} or antibiotics can be considered as indicative of increased nutritional needs under either thyroid condition.

Summary. 1. White Leghorn male and Rhode Island Red chicks of both sexes were fed a vit. B_{12}-deficient ration which was supplemented with 0.1% thiouracil and/or 0.5 to 4.0 μg % vit. B_{12} or 2 mg procaine penicillin G per lb of food. After a preliminary depletion period of one and 2 weeks, respectively, on the vit. B_{12}-deficient diet, the chicks were fed the above supplements for an additional 4 or 3 week period. 2. Thiouracil markedly reduced body weight gains and food intake in both breeds of chicks, and increased the grams of food required for each gram of gain in body weight. Vit. B_{12} and penicillin partially or completely counteracted this growth depression and increased appetite. The larger doses of vit. B_{12} were more effective in these respects than the smaller doses. 3. The large increase in thyroid weight and severe inhibition of comb growth induced by thiouracil were not influenced by the vitamin or antibiotic. 4. In general, these results corroborate previous data on the effects of vit. B_{12} and antibiotics on rats fed thiouracil or following parathyroidectomy.

---

1. Meites, J., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 193.
2. ———, *Metabolism*, 1952, v1, 58.
3. Greer, M. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 146.
4. Hartman, A. M., Dryden, L. P., and Cary, C. A., *Arch. Biochem.*, 1949, v23, 165.
5. Charkey, L. W., Wilgus, H. S., Patton, A. R., and Gassner, F. X., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 21.
6. Rupp, J., Paschkis, K. E., and Cantarow, A., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 432.
7. Libby, D., and Groschke, A. C., Unpublished observations.
8. Miller, D. C., and Groschke, A. C., *Mich. Agr. Exp. Sta. Quart. Bull.*, 1950, v32, 279.
9. Meites, J., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 195.
10. Meites, J., and Shay, J. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 196.
11. Meites, J., and Ogle, R. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 758.
12. Drill, V. A., *Physiol. Rev.*, 1943, v23, 355.

Received January 28, 1952. P.S.E.B.M., 1952, v79.

Volume of Distribution of Radiosulfate as a Measure of the Extracellular Fluid. (19382)

M. WALSER. (Introduced by A. Goth.)

From the Department of Internal Medicine, Southwestern Medical School, University of Texas.

Virtual equilibrium following the injection of certain tracer ions, such as Na^+ and Cl^-, requires several hours or days, presumably because exchange rates with some of the intracellular unlabelled ion are very slow(1,2). On the other hand, substances such as mannitol, which are largely excluded from body cells, may attain equilibrium throughout the extracellular fluid within 50 minutes, even though slow cell penetration occurs thereafter(3). Sulfate, as a consequence of its rapid capillary exchange rate(4), might be expected to attain equilibrium throughout the extracellular fluid even more rapidly. Studies with unlabelled sulfate indicated that this is the case(5).

Studies were undertaken of the distribution and excretion of radiosulfate in normal human subjects. S-35 has a convenient half-life of 87 days and can be administered to man without hazard.* A dose of 100 μc† was injected intravenously from calibrated 5 cc syringes during the course of one minute. Samples of serum or plasma drawn at intervals after the end of injection and an aliquot of the injected material appropriately diluted were enriched with sodium sulfate to minimize errors due to changes in self-absorption. Serum proteins were precipitated with trichloracetic acid. Sulfate was precipitated as the benzidine salt and collected on filter papers, which were permanently mounted on aluminum discs(6). Urine samples, obtained without the use of catheters, but with sufficiently great urine flow in most cases to minimize dead space errors, were analyzed similarly except that the amount of sulfate precipitated was determined by weighing each sample. Self-absorption corrections relative to the weight of the plasma samples were applied from an empirically determined curve. Radioactivity was measured by mica-window Geiger counter tube. Duplicates differed by an average of 0.7%.

Fig. 1 shows a composite concentration-time curve of plasma activity for 7 normal subjects. Values during the first 13 minutes were obtained from femoral arterial samples in order to reduce fluctuations due to intravascular mixing, in 4 of the 7 subjects.

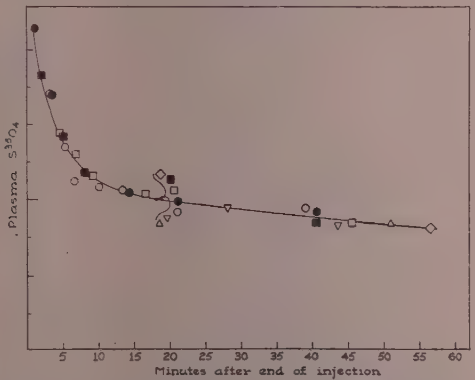


FIG. 1. A composite concentration-time curve of plasma activity for 7 subjects. For each subject (represented by a single type of symbol) curves were adjusted to the same 18 min value by appropriate factors. A curve of best fit for the entire group was drawn visually.

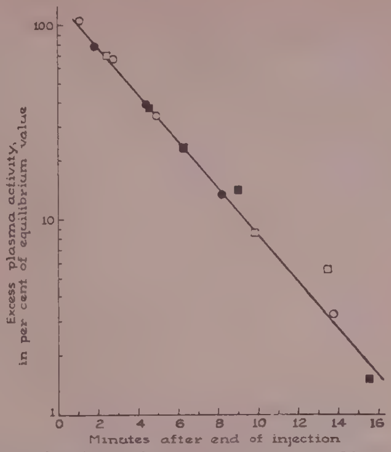


FIG. 2. Equilibration of radioactive sulfate. Excess plasma activity over the later slow rate of disappearance is plotted against time on a semi-logarithmic scale for four subjects. The line was drawn visually.

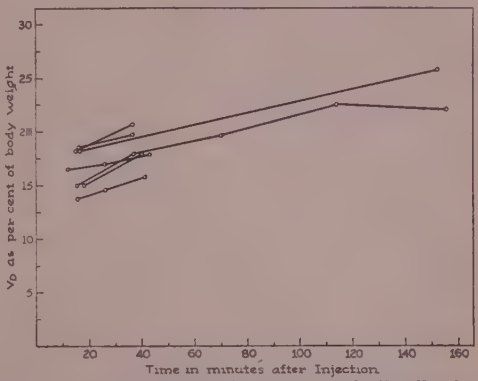


FIG. 3. The increase in volume of distribution (V_D) of radioactive sulfate with time in seven normal subjects. Volumes are expressed as % of body wt.

The curves for these 4 subjects were analyzed into 2 additive rates. This was done by extrapolating the later, straight part of the curve back to zero time on a semi-logarithmic plot and tabulating the differences between this line and the obtained values at each point. This difference is plotted semi-logarithmically against time in Fig. 2. For each subject a straight line with a decay constant of approximately 0.28 is obtained. The difference is plotted as per cent of the 18 minute value for

---

* 100 microcuries, if wholly retained in the body of a 70 kg man and eventually decayed there, would produce only 0.6 roengten equivalents physical.

† The S-35 labelled sulfate was obtained from the Oak Ridge National Laboratories on authorization by the Atomic Energy Commission.

TABLE I. Volume of Distribution at 18 Min of Radioactive Sulfate in 10 Normal Subjects.

Subject	Height, cm	Sex	Wt, kg	Vol of distribution, l	Body wt, %
H	173	♂	75	14	18.7
W	183	♂	78.8	13.4	17
MW	182	♂	70	10.6	15.1
B	175	♂	82.5	13.8	16.8
J	185	♂	84.6	13.8	16.3
N	170	♀	54.7	10	18.4
BL	164	♀	48.4	8.2	16.9
LS	162	♀	65.2	8.4	12.9
S	177	♂	79.6	12.2	15.3
JM	180	♂	43.3	6.8	15.7
				Avg	16.3

convenience in order to combine the results for all 4 subjects.

From 15-20 minutes on, the rate of fall of the plasma concentration is much slower (decay constants .003-.007).

In order to determine the extent to which urinary loss accounted for this later slow fall in the plasma concentration, the volume of distribution was measured at intervals following injection. It was calculated as the amount injected minus the amount excreted divided by the interstitial fluid concentration.‡ Eighteen minutes was selected as the first interval since the early rapid rate appeared to be completed at this time. The results are shown in Fig. 3. A progressive increase in the volume of distribution with time is noted in all subjects.

These results were interpreted to mean that radiosulfate becomes distributed throughout a certain compartment within 15-20 minutes, and slowly penetrates beyond it thereafter.

Two alternative hypotheses were considered: first, that this compartment represents only the more accessible portions of the extracellular fluid, and that penetration into the remainder of the extracellular fluid proceeds at a much slower rate; and secondly, that this compartment corresponds to the anatomic extracellular space and further penetration represents cellular uptake. A slow rate of uptake by bone and cartilage has been observed(7).

In order to attempt to answer this question the magnitude of the 18 minute space was determined in normal subjects. The results are shown in Table I. It should be noted that omission of corrections for plasma water and Donnan effect would increase each space 12%, changing the average value to 19.5% of body weight. The corrected mean of 16.3% body weight corresponds closely to the figure of 16% for ECF given in a recent review of compartment measurements by Levitt and Gaudino(8).

The constancy of this compartment was determined by serial measurements at intervals of several days in 6 individuals. In 9 out of 10 instances, the volumes checked within 0.3 liter.

These results tend to support the second hypothesis—that sulfate becomes distributed throughout the extracellular space within 15-20 minutes. It also seems improbable on *a priori* grounds that penetration into the interstitial fluid should proceed at 2 so vastly different rates, but this possibility cannot be excluded at present. Sheatz and Wilde reported rapid disappearance of radiosulfate in rats, but found volumes of distribution 34% of body weight 30 minutes after injection(4). The explanation of the discrepancy between this value for rats and the value for man reported here is not evident.

Summary. Radiosulfate becomes distributed within 15 to 20 minutes in a reproducible space which is 16% of the body weight in man. It is suggested that this space may correspond to the extracellular fluid.

---

‡ The plasma water concentration was divided by 0.90 to obtain interstitial fluid concentration. This is a reasonable approximation for the Gibbs-Donnan factor for divalent ions. No experimentally determined factor for sulfate was found in the literature.

1. Ray, C. T., Burch, G. E., and Threefoot, S. A.,

J. Lab. Clin. Med., 1951, v38, 940.

2. Levitt, M. F., and Gaudino, M., *Am. J. Physiol.*, 1949, v159, 67.

3. Houck, C. R., *Am. J. Physiol.*, 1951, v165, 102.

4. Sheatz, G. C., and Wilde, W. S., *Am. J. Physiol.*, 1950, v162, 687.

5. Bourdillon, J., and Lavietes, P. H., *J. Clin. Invest.*, 1936, v15, 301.

6. Henriques, F. C., Jr., Kistiakowsky, G. B., Margnetti, C., and Schneider, W. G., *Ind. Eng. Chem.* (Anal. Ed.), 1946, v18, 349.

7. Dziewiatkowski, D. D., *J. Biol. Chem.*, 1949, v178, 197.

8. Levitt, M. F., and Gaudino, M., *Am. J. Med.*, 1950, v9, 208.

Received January 28, 1952. P.S.E.B.M., 1952, v79.

Bromsulfalein Retention in Experimental Obstructive Jaundice. (19383)

Burton Giges, Joseph D. Mann, and William S. Sharon.
(Introduced by P. György.)

From the Department of Hepatic and Metabolic Diseases, Graduate School, Walter Reed Army Medical Center, Washington, D.C.

In a previous study, prolonged retention of bromsulfalein (BSP) was observed in patients with regurgitation jaundice(1). The present experiments were performed to study this phenomenon in the laboratory animal. The work of Snell, Greene, and Rowntree(2), and Cantarow and Wirts(3) suggested that phenoltetrachlorphthalein in the dog and bromsulfalein (phenoltetrabromphthalein) in the cat were cleared from the plasma rather rapidly despite complete biliary obstruction. These observations have been extended in the present study of BSP retention in dogs and rats with common bile duct ligation.

Material and methods. Six mongrel dogs were anesthetized with intravenous pentobarbital and the common bile duct of each was sectioned between 2 ligatures. Following complete recovery, BSP (5 mg per kilo) was injected intravenously. Tests were done at weekly intervals in each animal for an average of 3 weeks. Serum was analyzed for dye before each injection, and at ½, 24, 48 and 72 hours after the injection. The same operation was performed in 13 Sprague-Dawley rats under ether anesthesia. After recovery, 5 mg of BSP per 100 grams of body weight were injected intravenously or intraperitoneally. Serum obtained by cardiac puncture was analyzed at the same intervals as for the dogs. One test only was done in each rat. The method of Gaebler(4) using the Evelyn photoelectric colorimeter was employed in determining serum concentration of dye. Abnormal retention was considered present if the concentration exceeded 0.5 mg % in dogs(5) (equivalent to 5% retention), and 1.0 mg % in rats(6).

TABLE I. Duration of BSP Retention in Experimental Obstructive Jaundice.

	Dog*	Rat*
< 24 hr	9	0
24-48 hr	8	9†
48-72 hr	2	4‡

* No. of tests performed.
† Dye inj. intrap.
‡ " " intrav.

TABLE II. Average Daily Urinary Excretion of BSP in Experimental Obstructive Jaundice.*

Day	Dog	Rat
1	42	22
2	2	7
3	0	1
4	0	0
Total	44	30

* % of amount inj.

Results. In Table I, it will be seen that in dogs with complete biliary obstruction, with serum bilirubin values ranging from 8-12 mg %, BSP was removed from the serum within 72 hours. A similar rapid clearance was also observed in rats with common bile duct obstruction. The serum bilirubin in these animals was 5-6 mg %.

Analyses were carried out to determine how much of the retained dye was excreted. An analysis of the feces revealed no dye. This is not surprising since its presence in the stool requires a patent biliary system. In regard to renal excretion, Table II shows that in both species studied, less than 55% of the administered dye appeared in the urine.

Discussion. It is apparent that the results obtained in dogs and rats with regurgitation jaundice differ significantly from similar observations in human subjects(1). The rapidity with which BSP disappears from the serum of these animals suggests that other mechanisms are present for removal of dye in the absence of hepatic excretory function.

Cohn, Levine and Streicher showed that BSP is removed from the serum of the hepatectomized dog(7). Our data indicate that the dye is similarly removed when normal hepatic pathways are blocked. These data, however, are not applicable to BSP removal in man(1,8).

It has been stated that the kidney is able to excrete significant amounts of BSP when the serum concentration remains elevated for prolonged periods(9). In our experiments, 30-50% of the amount of dye given was recovered in the urine. Mechanisms of removal or destruction other than renal, therefore, probably play a role when the dye is not completely excreted through normal channels. In this connection, Brauer and coworkers have demonstrated that BSP may be chemically altered by the liver(10,11).

Summary. In dogs and rats with complete biliary obstruction, the retention of BSP in the serum was of much shorter duration than that previously observed in man. Conclusions concerning mechanisms of BSP removal in man based on observations made in the dog must be questioned in the light of these data.

---

1. Gigos, B., *J. Lab. Clin. Med.*, 1951, v38, 210.
2. Snell, A. M., Greene, C. H., and Rowntree, L. G., *Arch. Int. Med.*, 1925, v36, 273.
3. Cantarow, A., and Stewart, H. L., *Am. J. Path.*, 1935, v11, 561.
4. Gaebler, O. H., *Am. J. Clin. Path.*, 1945, v15, 452.
5. Unpublished observation.
6. Casals, J., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 383.
7. Cohn, C., Levine, R., and Streicher, D., *Am. J. Phys.*, 1947, v150, 299.
8. Mendeloff, A. I., Kramer, P., Ingelfinger, F. J., and Bradley, S. E., *Gastroenterology*, 1949, v13, 222.
9. Norcross, J. W., White, R. M., and Bradley, R. F., *Am. J. Med. Sci.*, 1951, v22, 137.
10. Brauer, R. W., Krebs, J. S., and Pessotti, R. L, *Fed. Proc.*, 1950, v9, 259.
11. Brauer, R. W., Pessotti, R. L., and Pizzolato, P., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 174.

Received February 4, 1952. P.S.E.B.M., 1952, v79.

Effect of Chloromycetin and Streptomycin on Embryonic Tissue Growth in *In vitro* Tissue Culture. (19384)

MATTHEW H. FUSILLO, JOSEPH F. METZGER, AND DWIGHT M. KUHNS.
(Introduced by Leland W. Parr.)

From the Laboratory Service, Walter Reed Army Hospital, Washington, D.C.

In a recent survey of the literature, it was noted that few reports have been presented concerning the effects of chloromycetin and streptomycin on normal cells in *in vitro* tissue culture. Ikegaki(1) has reported on the depressive effect of streptomycin on tissue growth, while Rodkova(2), Lepine, *et al.*(3) have presented conflicting data concerning chloromycetin.

Experiments were conducted in our laboratories with the following aims: (a) To satisfy increasing interest in the action of antibiotics on normal cells, with a view toward using antibiotics to help maintain sterility in tissue cultures. (b) To attempt to confirm the few existing reports.

Methods and Materials. Chloromycetin: Test solutions of chloromycetin were made up in the Tyrode portion of the usual tissue culture media(4) which was made up to the following concentrations in the final nutrient media: 1200, 900, 600, 480, 360, 240, 120 and

60 g/ml. *Streptomycin*: Test solutions of streptomycin sulfate were made in the same manner previously described in the following concentrations: 120000; 90000; 60000; 45-000; 30000; 15000; 6000; 4500; 3000; 1000; 600; 450; 300; 150; and 60 g/ml. *Tissue culture:* Tissue culture studies were conducted by the roller tube technic using a chicken plasma clot. Chicken plasma was obtained commercially (Difco), while the chick embryo juice was prepared in these laboratories. Chick embryo heart explants, cut into pieces about 1 mm square were obtained from 9-day-old chick embryos. The radius of growth about the explant was measured after 48 hours incubation at 37°C. The "average index of growth" listed in the tables is the average radius of new growth about the explants in the indicated groups with their respective standard errors. Duplicate experiments were conducted.

Results. Table I. *Chloromycetin.* Concentrations up to and including 360 g/ml had no significant inhibitory effect. A slight but consistent increase in growth was observed at 240 μg/ml, but this cannot be borne out statistically on the basis of the small number of cultures tested. Growth was reduced but not completely inhibited at concentrations of 480 μg/ml and higher. The latter substantiates the reports of Rodkova and Lepine.

TABLE I. Effect of Chloromycetin on Embryonic Cells in Tissue Culture. Growth index in relationship to chloromycetin free controls.

Conc. of chloromycetin, μg/ml	Avg index of growth* Exp. 1	Avg index of growth* Exp. 2	No. of explants
Controls	37.6 ± 5.2	64.7 ± 2.3	43
60	35.7 ± 4.2	65.7 ± 4.2	10
120	45.8 ± 8.2	60.6 ± 4.6	14
240	48.3 ± 8.1	72.5 ± 4.6	14
480	41.1 ± 4.4	55 ± 4.1	16
600	29.9 ± 3.8	52.5 ± 3.4	17
900	22.1 ± 1.4	15.7 ± 4.2	14
1200	20.4 ± .04	2 ± .04	13

* Index is average radius of new growth about the explants.

TABLE II. Effect of Streptomycin on Embryonic Cells in Tissue Culture. Growth index in relationship to streptomycin free controls.

Conc. of streptomycin, μg/ml	Avg index of growth* Exp. 1	Avg index of growth* Exp. 2	No. of explants
Controls	89.9 ± 4.6	46 ± 6.6	31
60	67.8 ± 5.8	45.1 ± 1.8	14
150	69.1 ± 5.4	Not read†	15
300	72.7 ± 6.4	Not read†	10
450	45 ± 6.2	43.3 ± 4.2	12
600	61.6 ± 4.2	47.8 ± 3.6	15
1500	64.4 ± 6	51.1 ± 3.6	16
3000	54.3 ± 3	39.1 ± 4.8	13
4500	58.3 ± 4	27 ± 6.4	13
6000	54 ± 2.6	Not read†	11
15000	17.7 ± 8.2	11.5 ± 3.2	13
30000	12.2 ± 2.1	5 ± 4	12
45000	2.2 ± 1.1	3 ± 2.4	13
60000	Not read†	3.3 ± 1	16
90000	No growth	No growth	17
120000	"	"	15

* Index is average radius of new growth about the explants.

† Explants not clotted.

Table II. *Streptomycin.* Growth was markedly reduced by concentrations of 6000 to 15000 μg/ml and was completely inhibited by a concentration of 90000 μg/ml. Generally this is in agreement with the results of Ikegaki.

Summary. Growth of normal embryonic tissue was completely inhibited by concentrations of 90000 and greater μg/ml of streptomycin. Growth was partially inhibited by concentrations of 480 μg/ml through the highest concentration of chloromycetin tested.

---

1. Ikegaki, Ken, *J. Antibiotics,* 1951, v4, 5.

2. Rodkova, H. K., *Casopis Lekarn Ceskych,* 1951, v90, 2.

3. Lepine, P., Barski, G., and Maurin, J., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 252.

4. Cornman, I., Personal communication.

Received February 4, 1952. P.S.E.B.M., 1952, v79

Thyroid and Adrenal Response to Varying Dose Levels of Epinephrine. (19385)

ALBERT L. BOTKIN AND J. T. TEW. (Introduced by H. Jensen.)

From the Army Medical Research Laboratory, Fort Knox, Ky.

It has become apparent that the release of the adrenocorticotropic and thyrotropic hormones for the anterior pituitary is dependent upon the physiological state and needs of the animal. The nature of the stimulus to the pituitary is still in question. The role of epinephrine in the release of these hormones has been considered by a number of workers (1-5). In the absence of quantitative data on the influence of epinephrine on the functional activity of the adrenal and thyroid, the present study was undertaken to determine and compare the simultaneous responses of these endocrine organs to varying dose levels of epinephrine.

Methods. Male rats of the Sprague-Dawley strain weighing 200-250 g were used. They were maintained on Purina laboratory chow and tap water until 18-24 hours before sacrifice, at which time they were deprived of food but allowed water *ad lib.* Two hours before sacrifice, each experimental animal was injected with 1.0 ml of epinephrine in 0.9% NaCl (containing 10, 20, 30, 50, or 100 μg of epinephrine.*), and each control was injected with 1.0 ml of saline. *Thyroid* I^{131} *Studies.* Four hours before sacrifice each rat was injected intraperitoneally with 1.0 ml of I^{131} solution containing approximately 5 microcuries of carrier free I^{131}† made to volume in phosphate buffer of pH 7.4. The animals were sacrificed by a sharp blow at the base of the skull and exsanguinated via a thoracic incision. The thyroid glands were removed, weighed and analyzed for their total and organic bound I^{131} contents according to the procedure of Botkin and Jensen(5). Control groups of 6 rats (saline injection) were run with each set of experimental animals (epinephrine injection). Groups of 12 or 24 experimental animals were used for each epinephrine dosage level. All values for I^{131} content were calculated as percent of the injected dose; those for the experimental animals are expressed as percentage deviation from control values and are illustrated in Table I.

TABLE I. Adrenal Cholesterol Content and Thyroid I^{131} Content After Epinephrine Injection. % deviation from control.

	μg epinephrine				
	10	20	30	50	100
Adrenal cholesterol	–6.2	–16	–19.9	–23.4	–30
Thyroid I^{131}	–5.7	–10.1	–15	–16.5	–24.1

Adrenal Cholesterol Studies. The animals were sacrificed by decapitation. Adrenals were removed, weighed and analyzed for total cholesterol by a modification of the method of Schoenheimer and Sperry(6). Control animals were run with each set of experimentals. Groups of 12 or 19 experimental animals plus 3 control animals were used for each epinephrine dosage level. All values were calculated as mg cholesterol per 100 mg adrenal wet weight; those for the experimental animals are expressed as percentage deviation from control values and are shown in Table I.

Results. Thyroid I^{131} *Studies.* Of the epinephrine dosages employed 10 μg produced a slight but not significant decrease in thyroid I^{131} content. With larger dosages there was an approximately linear decrease of I^{131} content of the gland from 10.1% below control values at 20 μg to 24.1% below control values at 100 μg (Table I).

Adrenal Cholesterol Studies. The decrease in adrenal cholesterol content observed after various levels of epinephrine injection paralleled very closely the degree of decrease found for thyroid I^{131} content. An insignificant decrease was produced by 10 μg. Progressively larger responses from 16.0% decrease at 20 μg to 30.0% decrease at 100 μg were found (Table I).

---

* Epinephrine Injection, U.S.P., 1:1000, Abbott Laboratories.

† The radioactive iodine(I^{131}) used in this investigation was supplied by the Oak Ridge National Laboratories on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

Discussion. The parallel responses of both thyroid I^{131} content and adrenal cholesterol content at the same short (2 hour) interval after administration of varying amounts of epinephrine might indicate a simultaneous stimulation of these endocrine organs. The question of whether the content of a hormone or its precursor in an endocrine organ is an accurate index of the amount of hormone released into the circulation must be considered in this connection.

The nature of the "epinephrine" stimuli which provoke an increased rate of release of adrenocorticotropin from the anterior pituitary has been actively investigated. Sayers(7) has expressed the view that this tropic hormone output from the anterior pituitary is regulated by the level of circulating target organ hormone. The metabolic effect of epinephrine may act to increase tissue utilization of the target organ hormones with a consequent lowering of venous titer of the hormones. Long and his associates(8,9) propose a biphasic mechanism whereby, in addition to regulation by the level of circulating target organ hormone, there is a direct stimulation of the pituitary by epinephrine.

It is possible that a mechanism of the type discussed above may also regulate the release of thyrotropic hormone from the anterior pituitary.

The apparent resulting increased release of both adrenocorticotropin and thyrotropin from the pituitary as suggested by the results of the present investigation probably cannot be maintained under all circumstances for an extended length of time but may give way to an increase of one at the expense of the other.

Summary. The smallest intraperitoneal dose of epinephrine which produces a significant response from the thyroid and adrenal glands of the rat in two hours (as measured by I^{131} concentration and cholesterol content respectively) is 20 μg. The responses observed with varying levels of epinephrine are quantitatively similar for the two glands and are approximately parallel between doses of 20 and 100 μg of epinephrine. This is interpreted as an increased rate of function of both glands. Various explanations of these findings are discussed.

1. Sayers, G., Sayers, M. A., White, A., and Long, C. N. H., Proc. Soc. Exp. Biol. and Med., 1943, v52, 200.

2. Long, C. N. H., and Fry, E. G., Proc. Soc. Exp. Biol. and Med., 1945, v59, 67.

3. Sayers, G., Sayers, M. A., Liang, T. Y., and Long, C. N. H., *Endocrinology*, 1946, v38, 1.

4. Soffer, L. J., Gabrilove, J. L., and Jailer, J. W., Proc. Soc. Exp. Biol. and Med., 1949, v71, 117.

5. Botkin, A. L., and Jensen, H., *Endocrinology*, in press.

6. Schoenheimer, R., and Sperry, W. M., *J. Biol Chem.*, 1934, v106, 745.

7. Sayers, G., *Physiol. Rev.*, 1950, v30, 241.

8. Gershberg, H., Fry, E. G., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. and Med.*, 1950, v23, 32.

9. McDermott, W. V., Fry, E. G., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. and Med.*, 1950, v23, 52.

Received February 8, 1952. P.S.E.B.M., 1952, v79.

Serum Histaminase During Rabbit Anaphylaxis.* (19386)

B. Rose and J. Leger. (Introduced by J. S. L. Browne.)

From the McGill University Clinic, Royal Victoria Hospital, Montreal.

Rose and Weil(1) showed that anaphylaxis in the rabbit was accompanied by a sudden and marked decrease of blood histamine, and this was subsequently confirmed(6). Additional studies(2) showed that the decrease in histamine was confined not only to the blood, but occurred in tissues such as lung, liver and spleen as well. Because of the striking dis-

---

* These studies were supported by a grant from the John and Mary R. Markle Foundation. Part of the expenses were defrayed by a grant from the CIBA Co., Montreal.

appearance of naturally occurring histamine from these sites, it was decided to investigate the ability of this mechanism to deal with injected histamine. It was first noted that histamine injected either intravenously or by the intraperitoneal route produced an increase in the plasma histamine content which gradually disappeared over a period of minutes. If, however, the histamine was injected into previously sensitized rabbits, a marked and sudden disappearance of not only the preexisting but also of the injected histamine occurred following the injection of a shock dose of antigen(2). These findings made it clear that some powerful mechanism for the inactivation or "detoxification" of histamine was stimulated by the induction of anaphylaxis in this species. The present investigation was carried out in order to elucidate the nature of the mechanism involved.

Methods. Nineteen rabbits weighing between 2.5 and 3 kg each were sensitized to egg albumen. The egg albumen (Merck) was made up to a 3% solution in normal saline, and filtered before using. Each animal received 2 cc intraperitoneally every second day until five doses had been given. Two to three weeks after the last sensitizing dose, anaphylaxis was induced by the intravenous injection of 2 cc of antigen. The blood samples were taken by cardiac puncture both before and about 3-4 minutes after the injection of the shock dose when signs of anaphylaxis such as prostration, defecation and micturition were present. Each sample consisted of 20 cc, two of which were used immediately for histamine determination of the whole blood, and the remainder, after clotting, for histaminase analysis of the serum. The blood histamine was determined by the Code (3) modification of Barsoum and Gaddum's method(4), and is expressed as the base as γ/cc. The method of Ahlmark(5) was used for the histaminase determinations. Serum histaminase is expressed as γ histamine destroyed per cc plasma per hour. All determinations were done in duplicate.

Results. The results are given in Table I. The blood histamine values are in the left hand columns, and show the total blood histamine before and after the induction of shock. It will be seen that in all animals, with the exception of rabbit 19, a sharp decrease occurred. These results are confirmatory of those previously reported(1,2). In the right hand columns, the serum histaminase values are presented for the corresponding animals before and after the induction of anaphylaxis. Here it will be noted that a moderate to marked increase in activity of the serum histaminase was produced. Again, the one exception is in rabbit 19. The average control serum histaminase was 0.41 γ/cc/hr whereas following the induction of anaphylaxis the activity was 0.87 γ/cc/hr, which is an increase of 126%.

TABLE I. Rabbit Blood Histamine and Serum Histaminase Before and After Induction of Anaphylactic Shock in 19 Rabbits.

Blood histamine, γ/cc		Serum histaminase, γ/cc/hr		
Control	During shock	Control	During shock	% increase
—	.17	.55	.88	60
—	.22	.27	.75	178
—	.37	—	.98	—
—	.35	.37	1.25	238
2.1	.3	.37	1.12	200
—	—	.26	.84	222
1.73	.2	.26	1.06	308
3.6	.525	.39	.47	20
1.87	.17	.31	.83	68
1.5	.35	.43	.78	81
3.2	.25	.53	1.62	203
3.4	.55	.53	.66	24
3.8	.4	.49	.87	78
3.6	.4	.67	.81	21
3	—	.59	.80	35
2.88	.5	.49	1.34	174
4	.55	.29	.83	186
3.6	.4	.49	.78	59
4.6	4.4	.20	.18	0
	Avg	.41	.87	126

Signs of anaphylaxis were produced in the majority of animals, with death occurring in 12. There appeared to be no correlation between the intensity of the shock and the degree of change either in the blood histamine or serum histaminase values after induction of shock. The one exception was animal 19, in which there were no signs of shock, and little alteration of the blood histamine or serum histaminase values. It is also apparent that the reduction in blood histamine was more consistent in degree than the corresponding increase in serum histaminase.

Discussion. These results support the view that the detoxification or inactivation of histamine which occurs following the induction of anaphylaxis in the rabbit may be due in part to an increase in the activity of the enzyme histaminase. It is likely that the source of this increased histaminase activity is from one of the parenchymal organs such as lung or kidney. Dragstedt and his co-workers(6) showed that when antigen was added to rabbit blood which was being perfused through isolated sensitized lung, there occurred a decrease in the blood histamine content. The histamine was probably being destroyed in the lung tissue although histaminase determinations of the tissue itself were not made. More recently, Carlsten *et al.*(7) have observed a high histaminase activity of lymph in dogs, the main source of which Carlsten(8) found to be kidney and gut. It is probable that another major factor contributing to the disappearance of blood histamine during rabbit anaphylaxis is the equally rapid disappearance of the white cell elements which takes place at the same time(2), since the bulk of blood histamine is contained in these cells in an inactive form (9). As discussed elsewhere(10), the production of anaphylaxis in the rabbit must be due to a release of histamine from cells into the plasma and not simply to the disappearance of histamine which occurs, for no symptoms occur when glycogen is injected intravenously into the rabbit although a rapid disappearance of blood histamine occurs(11). This is supported by the finding that addition of glycogen to rabbit blood *in vitro* does not alter the distribution of histamine, whereas the addition of antigen to sensitized rabbit blood *in vitro* causes a shift of histamine from the cells into the plasma(11).

Summary. 1. Studies on the blood histamine and the serum histaminase were made in a series of 19 rabbits before and after the induction of anaphylactic shock. 2. In all but one, a reduction of the blood histamine was produced following anaphylaxis. This confirms previous observations. 3. An increase in the serum histaminase activity was noted in all animals except the one in which there was no reduction of the blood histamine. 4. These findings support the view that the rapid disappearance of blood histamine which occurs during anaphylaxis in this species may be due in part to an activation of histaminase.

We wish to thank Mrs. E. V. Harkness for technical assistance.

---

1. Rose, B., and Weil, P., PROC. SOC. EXP. BIOL. AND MED., 1939, v42, 494.
2. Rose, B., *J. Immunol.*, 1941, v42, 161.
3. Code, C. F., *J. Physiol.*, 1937, v89, 257.
4. Barsoum, G. S., and Gaddum, J. H., *J. Physiol.*, 1935, v85, 1.
5. Ahlmark, A., *Acta Physiol. Scandinav.*, 1944, v9, 27.
6. Dragstedt, C. A., de Arellano, M. R., and Lawton, A. H., *Science*, 1940, v91, 617.
7. Carlsten, A., Kahlson, G., and Wicksell, F., *Acta Physiol. Scandinav.*, 1949, v17, 370.
8. Carlsten, A., *Acta Physiol. Scandinav.*, 1950, v20, 5.
9. Code, C. F., *J. Physiol.*, 1937, v9, 349.
10. Rose, B., *Am. J. Med.*, 1947, v3, 545.
11. Rocha e Silva, M., Grana, A., and Porto, A., PROC. SOC. EXP. BIOL. AND MED., 1945, v59, 57.

Received February 11, 1952. P.S.E.B.M., 1952, v79.

Effect on Urinary Nitrogen Excretion of Rate of Administration of Solution of Amino Acids in Man. (19387)

Charles S. Davidson and Richard D. Eckhardt.*

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services, Boston City Hospital, and Department of Medicine, Harvard Medical School.

A protein hydrolysate has been prepared for intravenous use in man which is virtually free of the dicarboxylic amino acids, aspartic and glutamic acids(1). The significant reduction in anorexia, nausea, and vomiting from this solution compared to preparations containing these amino acids has been accompanied by an increase in possible rate of infusion without reaction, thus broadening the clinical usefulness and patient comfort in parenteral feeding(2). With this solution, rapid rates of infusion are possible, in fact up to 10 mg of nitrogen/kg/minute intravenously, representing an infusion time of just over 10 minutes for 500 cc of a 10% solution. Such rapid rates of infusion raise the possibility of increased urinary excretion of the infused amino acids or increased conversion to urea. Previously rapid rates of infusion induced greater urinary excretion of amino acids; nevertheless even at very rapid rates only about 10% of the 50 g infused was excreted(3). The urinary excretion of amino acids correlated more closely with size of infusion than with rate of administration. There was no selective excretion of any one of the 10 essential amino acids determined microbiologically.

The data presented in this communication were collected to determine whether rapid rates of infusion of the amino acid solution resulted in increased deamination of amino acids and hence increased excretion of non-protein nitrogen in the urine.

Materials and methods. The amino acid mixture† was made by the complete acid hydrolysis of casein, contained no peptides, was essentially devoid of the dicarboxylic amino acids (glutamic and aspartic), and was supplemented with dl-tryptophane and glycine so that it contained approximately 10% amino acids including the 8 essential for man. Seven subjects were selected. Two were normal medical students, 3 had liver disease (alcoholic cirrhosis), mild in 2 and severe in one, one patient had severe undernutrition from prolonged obstruction due to carcinoma of the larynx, and one was a chronic alcoholic essentially normal metabolically at time of study. The 24-hour urine was collected in glacial acetic acid and toluol and the total nitrogen content determined by micro-Kjeldahl digestion and Keyes' distillation. All urines were examined for protein and analyzed for alpha amino nitrogen by the gasometric ninhydrin method as described by Van Slyke, MacFadyen, and Hamilton(4). The patients were maintained on the ward with nurses and dietitians in attendance trained in metabolic methods. The food consumed daily was calculated from standard tables.

Results. Fig. 1 and 2 show the intake of protein by mouth and of amino acids intravenously, the total nitrogen excreted in the urine, and the rate of administration of the 10% solution of amino acids in terms of mg of nitrogen per kg per minute. Although nitro-

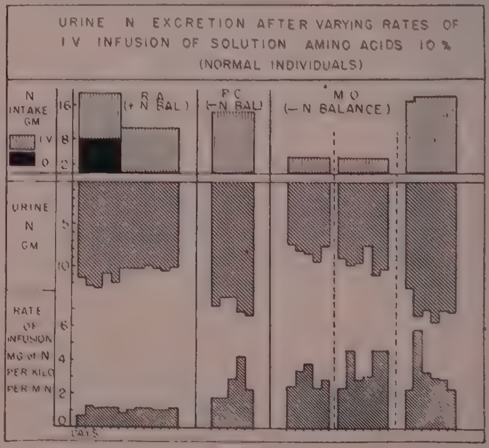


FIG. 1.

---

* Present address: State University of Ia., University Hospitals, Iowa City.

† Furnished by Merck & Co., Rahway, N. J.

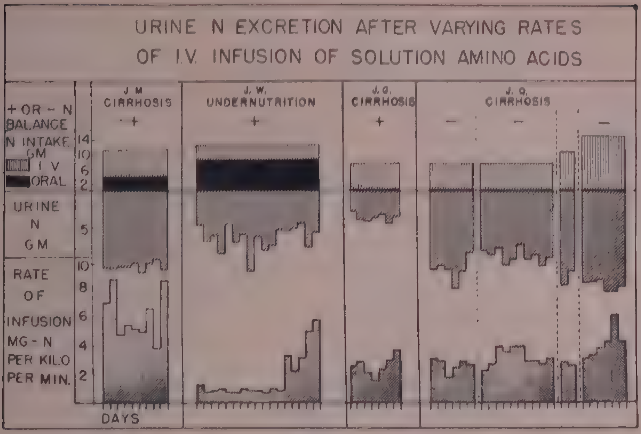


FIG. 2.

gen balance was performed on these patients, it is indicated in the chart only by the + (positive) or — (negative) sign. In four patients nitrogen balance was positive throughout, while in 3 negative balance obtained since for concurrent studies the total nitrogen intake was maintained below the minimum requirement.

The nitrogen excretion varied greatly from individual to individual depending upon nitrogen intake, nutritional status, and other factors. There was also day to day variation in nitrogen excretion in individual patients. There was a wide variation in the rate of administration of the solution from below 1 mg/kg body weight/minute to almost 9. In spite of this wide variation, there was no correlation between rate of administration and quantity of nitrogen excreted in the urine during each 24-hour period, either from patient to patient or in the individual patients. This is well illustrated by patient J.W. (Fig. 2) in whom there was little variation in nitrogen excretion although the rate of administration varied from 1 to almost 6 mg nitrogen/kg/minute.

The urinary excretion of alpha amino nitrogen was determined daily and formed only a small portion of the total urinary nitrogen. This fraction did not exceed 10% of the total nitrogen excreted, and, as previously observed (3), did not increase strikingly with marked increases in the rate of administration of the solution. Proteinuria did not occur. The variations in the excretion values for urinary total nitrogen shown in Fig. 1 and 2, therefore, reflect primarily changes in the urinary excretion of non-protein, non-amino acid nitrogen.

Discussion. The advantages of the 10% solution of amino acids used in this investigation are chiefly those of the low incidence of anorexia following infusion and the possibility of rapid rates of administration without nausea and vomiting. Although it was previously shown that even rapid rates of infusion did not increase the excretion of amino acids sufficiently to limit the nutritional value of the material for intravenous protein feeding(2), the possibility that increased deamination might occur, thus limiting its biological value, had to be considered. This was especially true since Silber and Porter(5), using the same or a similar preparation in dogs, observed that a 4-fold increase in the rate of infusion caused a decrease of approximately 60% in nitrogen retention, not, however, due to an increased loss of amino acids but rather to non-amino acid, non-protein nitrogen. The rates of infusion used by Silber and Porter usually were greater per kg in their dogs than in the individuals reported here. However, even in the few instances in which a similar rate was used in our patients, for example in patient J.M., no increase in nitrogen excretion occurred so that there was no change in nitrogen retention or biological value. In fact, there appeared to be no influence of rate of infusion on nitrogen retention whether the amino acid mixture was given as a sole source of nitrogen or as a supplement to small or large protein intakes by mouth.

Summary. 1. Seven subjects (including normal individuals and patients) were given daily intravenous infusions of a 10% solution of amino acids at widely varying rates of infusion. 2. There was no correlation between the daily non-protein nitrogen excretion and the rate of infusion. Very rapid rates of infusion did not increase the nitrogen excretion and thus did not reduce the biological value of the amino acid solution.

---

1. Howe, E. E., Unna, K., Richards, G., and Seeler, A. O., *J. Biol. Chem.*, 1946, v162, 395.

2. Eckhardt, R. D., and Davidson, C. S., *New England J. Med.*, 1948, v239, 164.

3. Eckhardt, R. D., and Davidson, C. S., *J. Clin. Invest.*, 1948, v27, 727.

4. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, 1943, v150, 251.

5. Silber, R. H., and Porter, C. C., *J. Nutrition*, 1949, v38, 155.

Received September 13, 1951. P.S.E.B.M., 1952, v79.

Protective Effect of Pitressin and of Epinephrine Against Total Body X-Irradiation. (19388)

John L. Gray, Elizabeth J. Moulden, John T. Tew, and H. Jensen.

From the Army Medical Research Laboratory, Fort Knox, Ky.

Pendergrass and his associates(1) have demonstrated a protective effect of pitressin and epinephrine against local x-irradiation injury of the leg of the rat. It was assumed by the authors that local tissue anoxia, produced by these principles, was involved in the protective mechanism. It became of interest, therefore, to investigate the possible effect of these agents on the aerobic and anaerobic metabolic cycles, as well as to study the effect of these agents on the survival rate after total body x-irradiation.

Methods. "Labile phosphate" (ATP*) content and cytochrome oxidase activity of muscle of rats as influenced by pitressin and epinephrine administration or by limb occlusion. Phosphate content and cytochrome oxidase activity determinations were done on gastrocnemius muscle samples from normal male Sprague-Dawley rats weighing 220-300 g. The rats were starved for 24 hours with water *ad libitum*, and were sacrificed 15 or 30 minutes following intraperitoneal injection of pitressin (Parke, Davis) or epinephrine (Abbott). Control animals received equivalent volumes of 0.9% saline or no treatment prior to sacrifice. Treated and untreated controls showed no significant difference and were grouped in the calculations. Phosphate determinations on lyophilized tissue were carried out according to the method described by Umbreit(2). Cytochrome oxidase activity was determined according to the method of Schneider and Potter(3). In the occluded limb experiments the rats were anesthetized with sodium pentobarbital (45 mg/kg) and a tourniquet consisting of 5 turns of a rubber band (Eberhard Faber No. 30) was applied to the right hind leg as high as possible. The teeth were clipped to prevent tourniquet removal or self-laceration. Control animals were anesthetized and the teeth were clipped. All animals were sacrificed without tourniquet release at ½ hour or 4½ hours after tourniquet application. Gastrocnemius muscle samples were taken from the occluded limb as well as from the opposite hind limb of the tourniquet animal and from a hind limb of the control animal. The effect of pitressin or epinephrine administration against total body x-irradiation: Male Sprague-Dawley rats weighing 220-300 g were irradiated in pairs,

---

* Adenosinetriphosphate.

TABLE I. Effect of Pitressin and Epinephrine on Muscle Phosphorus.

Treatment	Sacrificed, min	No. of rats	Phosphorus, avg ± S.D. Inorganic, mg % dry wt	Phosphorus, avg ± S.D. Labile, mg % dry wt
Control		40	42.2 ± 8.2	211.2 ± 24.9
Pitressin 5 units IP	15	12	49 ± 8.9 $p = .1$	248.7 ± 29.2 $p < .05$
	30	12	51.7 ± 9.3 $p < .05$	229.4 ± 27.9 $p < .2$
Epinephrine .2 mg IP	15	12	32.7 ± 8.1 $p < .05$	165.9 ± 27.5 $p < .05$
	30	12	34.5 ± 6 $p < .05$	188.3 ± 47 $p = .2$

$p < .1$ considered statistically significant.

TABLE II. Effect of Tourniquet Occlusion on Muscle Phosphorus. Average values ± S.D.

Exp. conditions	No. of rats	Inorganic P, mg % dry wt	Labile P, mg % P dry wt	Inorganic P / Labile P
Tourniquet on ½ hr—sacrificed without release				
Control—no tourniquet	6	39.2 ± 4.3	187.5 ± 18.2	.21 ± .03
" leg-tourniquet animal	10	49.5 ± 7.7	187.9 ± 18.5	.27 ± .04
Occluded leg- " "	10	77.5 ± 11.9	259.1 ± 24.7	.31 ± .07
Tourniquet on 4½ hr—sacrificed without release				
Control—no tourniquet	5	34.9 ± 1.3	183.4 ± 21.1	.19 ± .03
" leg-tourniquet animal	10	44 ± 9	188.9 ± 24.7	.24 ± .05
Occluded leg- " "	10	115.9 ± 20.1	361.3 ± 37.8	.32 ± .07

one serving as a control, the other as a treated animal. Each pair was exposed to total body x-irradiation for 22 minutes in a single exposure. Radiation factors were: 200 kv, 6 ma, ½ mm Cu 1 mm Al filter, target distance approximately 29 cm, and 40 r/min. dosage rate measured in air.[†] Five units (0.25 ml) of pitressin or 0.2 mg (0.2 ml) of epinephrine were injected intraperitoneally either 5, 20, or 40 minutes before or 5 minutes after exposure. Animals were housed in individual cages and weighed daily until death or termination after 28 days.

Results. As can be seen from Table I, following pitressin injection the inorganic and "labile phosphate" (ATP) values for muscle tissue were elevated at 15 minutes. At 30 minutes the "labile phosphate" was slightly elevated but the difference from the control was no longer significant. On the other hand, epinephrine administration produced at 15 minutes a lowering of the inorganic and "labile phosphate" (ATP) content of muscle tissue. Again the "labile phosphate" difference at 30 minutes was no longer significant. Preliminary investigations have indicated that cytochrome oxidase activity of muscle tissue was slightly but definitely decreased following pitressin administration and increased following epinephrine injection.

TABLE III. Effect of Tourniquet Occlusion on Muscle Cytochrome Oxidase. Average values±S.D. Sacrificed without release of tourniquet.

	No. of determinations	Q_{O_2}
Tourniquet on ½ hr		
Control—no tourniquet	8	300 ± 58
" leg-tourniquet animal	8	359 ± 87
Occluded leg- " "	8	289 ± 66
Tourniquet on 4½ hr		
Control—no tourniquet	6	276 ± 49
" leg-tourniquet animal	6	301 ± 74
Occluded leg- " "	6	173 ± 61

To demonstrate the possible effect of anoxia on muscle phosphate content and cytochrome oxidase activity, these constituents were determined in rat muscle tissue of tourniquet-occluded limbs. In local x-irradiation studies, Pendergrass and his associates(1) found that the greatest increase of radiation threshold was obtained with tourniquet-occlusion of the exposed limb.

Table II illustrates that occlusion of the limb for ½ hour and for 4½ hours produced a pronounced increase in the inorganic and

---

[†] The authors wish to express their appreciation to the Radiobiology Branch of this Laboratory for advice and assistance in the irradiation procedure.

TABLE IV. Effect of Pitressin and Epinephrine on Survival of Rats Exposed to X-Radiation.

Treatment	Time of treatment relative to x-radiation, min	Total roentgens	No. of rats	Survival							
				1st wk		2nd wk		3rd wk		4th wk	
				No.	%	No.	%	No.	%	No.	%
P,* 5 units IP	5 before	880	42	39	93	36	86	36	86	36	86
C*			43	25	58	6	14	5	12	5	12
P, 5 units IP	”	1000	18	14	78	9	50	8	44	7	39
C			18	2	11	0	0	0	0	0	0
P, 5 units IP	”	1120	19	10	53	6	32	5	26	5	26
C			14	0	0	0	0	0	0	0	0
P, 5 units IP	20	880	31	29	94	22	71	20	65	19	61
C			31	11	35	4	13	3	10	3	10
P, 5 units IP	40	880	26	23	88	15	58	12	46	12	46
C			27	15	56	7	26	6	23	6	23
P, 5 units IP	5 after	880	29	24	83	12	41	11	38	11	38
C			31	18	58	8	26	7	23	7	23
E,* .2 mg IP	5 before	880	34	29	85	20	59	20	59	20	59
C			34	19	56	8	24	8	24	8	24

* P = Pitressin, E = Epinephrine, C = Control.

"labile phosphate" (ATP) content of the muscle tissue of the tourniquet-occluded limb. Cytochrome oxidase activity was found to decrease gradually, depending on the length of time the limb was occluded (Table III). After ½ hour occlusion, no significant change was observed. However, with 4½ hours occlusion, the occluded limb showed a significant decrease in cytochrome oxidase activity.

As an extension of these findings, a study was made of the possible protective effect of pitressin and epinephrine against total body x-irradiation. The effect of pitressin or epinephrine on mortality after total body x-irradiation is illustrated in Table IV. As would be expected with an 880 r dose in total body exposure, the majority of deaths occurred between 6 and 14 days. Pitressin, given 5 minutes before irradiation, afforded considerable protection with a survival rate of 86% as compared with 12% survival for the untreated irradiated group. Similarly, pitressin injected 20 minutes before exposure decreased the mortality significantly. With injection 40 minutes prior to irradiation the protection was diminished but still definite. The beneficial effect of pitressin pretreatment was further indicated by the observation that the surviving pitressin-treated irradiated animals lost less weight and recovered weight very rapidly compared with irradiated control animals. *Epinephrine,* administered 5 minutes before irradiation, also seemed to give protection, but apparently not to the same degree as pitressin. *Pitressin,* given 5 minutes after irradiation, would appear to give only very slight, if any, protection. *Threshold studies,* employing greater x-ray dosages, indicate that with pitressin injection 5 minutes before irradiation the threshold could be raised from about 800 r to about 1100 r.

Discussion. The protective effect of pitressin and epinephrine against local (leg) and total body x-irradiation injury may be due to the tissue anoxia produced by these principles, leading to a reduction in the oxygen content of the tissue fluid.

The phosphorus and cytochrome oxidase activity results are interpreted as indicating that administration of either pitressin or epinephrine produces a temporary local tissue anoxia. The mechanism producing the anoxia differs for the two substances.

Geiling and DeLawder(4) found that after the injection of pitressin the muscles pass for a short time into a state suggesting that their activity was being carried out under anaerobic conditions. The blood coming from the muscles was arterial in color; it had a low CO_2 and a high O_2 content. The pitressin-induced tissue anoxia with the consequent disruption of the aerobic cycle leads to an increase in the rate of glycolytic processes followed by increased formation of ATP. The results obtained with tourniquet-occluded limbs are similar to those obtained after administration of pitressin, and substantiate the explanation given for the effect of pitressin.

It is known that an immediate but short-lived increase in the oxygen consumption follows the administration of epinephrine. The mechanism of this action is not known. Epinephrine, by causing increased cell activity (oxygen consumption), brings about an increased utilization of ATP leading to a lowering of the "labile phosphate" content. The increase in the oxygen consumption following epinephrine administration and the vasoconstrictive action of epinephrine, decreasing the tissue supply of oxygen, may lead to a temporary tissue anoxia.

It is generally assumed that many of the biological effects of x-irradiation can be attributed to the action of the decomposition products of water which result from the irradiation. It is also becoming increasingly evident that the degree of x-radiation effects is, to an important extent, dependent on the oxygen content in the cellular fluids. Dowdy, Bennett and Chastain(5) exposed rats in very low oxygen tension (5%) and found that the acute effects of radiation are only half as severe. Remarkable decreases in many x-ray induced cellular phenomena by reduction of oxygen concentration during irradiation have been reported(6,7).

Summary. 1. The survival rate of rats, exposed to lethal x-ray dosage, was found to be significantly increased after pretreatment with pitressin or epinephrine. 2. The protective effect of these principles may be due to their property of producing a temporary tissue anoxia.

---

1. Pendergrass, E. P., personal communication.
2. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Technics and Related Methods for the Study of Tissue Metabolism. Minneapolis, Burgess Publishing Co., 1945.
3. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, v149, 217.
4. Geiling, E. M. K., and Delawder, A. M., *Bull. Johns Hopkins Hosp.*, 1932, v51, 1 and 355.
5. Dowdy, A. H., Bennett, L. R., and Chastain, S. M., *Radiology*, 1950, v55, 879.
6. Horowitz, N. H., and Mitchell, H. K., *Ann. Rev. Biochem.*, 1951, v20, 469.
7. Curtis, H. J., *Ann. Rev. Physiol.*, 1951, v13, 45.

Received December 3, 1951. P.S.E.B.M., 1952, v79.

Comparative Antigenic Potency of Killed Irradiated Tubercle Bacilli and Living BCG Vaccine. (19389)

KONRAD BIRKHAUG AND RAFAEL DARRICARRERE.*

From the Division of Laboratories and Research, New York State Department of Health, Albany, N. Y.

Criticism is raised against artificial immunization of man with living BCG vaccine on the assumption that the organisms may survive for years and gradually regain virulence(1). The U. S. Public Health Service(2) and the American Trudeau Society(3) recommend further research to develop a tuberculosis vaccine containing only dead organisms. It is well known from the experimental and clinical studies(4) that such vaccines hitherto have yielded less effective protection against a challenge virulent infection than living BCG vaccine. The question has arisen whether or not the prolonged heating required to kill tubercle bacilli vitiates against their antigenic potency. In order to eliminate this factor, vaccine has recently been prepared by killing virulent human tubercle bacilli by brief ultraviolet irradiation(5-7). Such vaccines are reported to be equal or superior to living BCG vaccine on the basis of guinea pig and mouse protection tests. The advantages of a killed tuberculosis vaccine are twofold since it would be absolutely safe and remain relatively stable upward to one year while the optimal usefulness of living BCG vaccine is approximately 10 days and one fears that it may regain virulence. For the purpose of evaluating the comparative antigenic potencies of killed irradiated and living BCG vaccines we undertook the following study.

---

* Fellow of U. S. Public Health Service and Chief of the Tuberculosis Department of the Bacteriological Institute at Santiago, Chile.

Methods. Two lots of killed irradiated tuberculosis vaccine were kindly supplied us by R. W. Sarber(6) and Dr. A. Milzer(7). These were prepared 15 and 3 months previously with human type tubercle bacilli, strains H37Rv and 199RB and contained 1 mg of organisms per ml. A 1:10,000 dilution of merthiolate was added to each lot as preservative. The vaccines had been cleared for sterility and safety in guinea pigs and mice. Following the procedure detailed by Sarber (6), the H37Rv irradiated vaccine was administered intraperitoneally in three 5 ml (5 mg of organisms) doses at weekly intervals to 24 normal and tuberculin-negative guinea pigs weighing 421 ± 27.61 g.[†] Similar inoculations with freshly prepared(8) living BCG vaccine (Lots B-110, A-111 and B-111 currently distributed for use in man in New York) were made in another lot of 24 normal and tuberculin-negative guinea pigs weighing 431 ± 35.99 g. One mg of these respective lots of BCG vaccine was found to contain 46, 56, and 54 million living elements (colonies) in Löwenstein cultures. Two similar groups of guinea pigs weighing respectively 436 ± 25.75 and 426 ± 25.68 g were given identical inoculations with 199RB irradiated vaccine and living BCG vaccine (Lots A-115, B-115, and A-116), the latter containing 58, 65, and 51 million living elements (colonies) per mg of organisms. The first series of 48 animals was allowed an immunization period of 3 months, and the second series of 1 month after the last intraperitoneal inoculation. At this time, both series of immunized animals, together with 18 normal and tuberculin-negative control guinea pigs weighing 620 ± 15.17 g, were given 0.005 mg of a 3-week-old Löwenstein culture of H37Rv (of established virulence) by the intramuscular route in the right hind leg. This dose gave rise to 2,862 eugonic colonies on a series of Löwenstein cultures after 12 weeks' incubation. At the time of the challenge virulent inoculation, no significant difference in the body weight obtained between the first series of immunized animals and the controls while a difference of approximately 100 g existed between the second series of immunized animals and the controls.

Results. All the animals inoculated intraperitoneally with either lot of irradiated vaccine experienced extreme irritability and distress immediately following the inoculations, a phenomenon not observed in the animals similarly inoculated with BCG vaccine. We attributed this occurrence to the presence of merthiolate in the irradiated vaccine while BCG vaccine contained no preservative. The post-immunization mortality rate was greater in the animals given the irradiated vaccine than in the BCG vaccine group. Within 4 weeks after the last immunizing dose, 7 and 6 animals (27.1%) died respectively in the first and second series of irradiated vaccine groups and 2 in each of the BCG vaccine groups (8.3%).

Post-immunization tuberculin reactions 48 hours after the intradermal inoculation with 0.0001 mg PPD showed greater induration volumes in the animals inoculated with BCG vaccine than with irradiated vaccine in tests performed at bi-weekly intervals after immunization (Table I presents data on tests done 1 month after immunization). After the challenge virulent inoculation, we observed in both series of animals a more retarded increment in tuberculin hypersensitiveness in the animals immunized with BCG vaccine than with either lot of irradiated vaccine. Table I gives data on tests done 3 months after the challenge inoculation. We also ascertained by palpation a much slower development of lymphonodular hypertrophy in the vicinity of the virulent inoculation in the animals immunized with BCG than with either lot of irradiated vaccine.

Approximately 3 months after the challenge virulent inoculation, the body weight of the BCG immunized animals was 780 ± 90.22 g and 716 ± 78.10 g respectively in the first

---

[†] ± means stand. dev. of weights of animals.

TABLE I. Post-Immunization and Post Challenge Findings in Guinea Pigs Inoculated with Killed Irradiated Tubercle Bacilli and Living BCG Vaccine.

Material	First series		Second series		
	Irrad. tbc. 1	BCG	Irrad. tbc. 2	BCG	Controls
No. animals at start	24	24	24	24	18
Post-immunization deaths	7	2	6	2	—
Post-challenge deaths	5	8	6	4	8
Induration (mm^3) with .0001 mg PPP after 48 hr					
1 mo after immunization	426 ± 109	760 ± 403*	422 ± 121	572 ± 198*	—
3 ” ” challenge	2953 ± 1598	1910 ± 319*†	2184 ± 1148*	1085 ± 298*†	2850 ± 1511
No. animals autopsied	17	22	18	22	18
Wt at autopsy, g	698 ± 117	789 ± 90*	624 ± 94	710 ± 78*	617 ± 64
Completely protected	0	13	0	12	0
Localized tuberculosis	9	4	10	4	2
Generalized ”	8	5	8	6	16
Rt. sup. inguinal glands, g	.89	.32*	.54*	.24*	1.41
” deep ” ” ”	.30*	.16*	.32	.10*†	.59
” iliac ” ” ”	1.46	.42*†	1.17	.34*†	2.00
Pooled 17 sets ” ”	9.45	3.10*†	8.29*	2.14*†	13.47
Spleen ”	2.50	1.00*†	1.78*	1.20*	3.22
Liver ”	37.32	34.30*	36.33	32.50*	41.39
Lungs ”	7.86*	5.94*†	6.37	6.13*	9.58

* Difference between control and these figures } significant by:
† ” ” BCG and irrad. tbc. figures }

± is stand. dev. by $\sqrt{\frac{S(d^2)}{n-1}}$.

$\underline{t} = \frac{x-x'}{s}\sqrt{\frac{(n_1+1)(n_2+1)}{n_1+n_2+2}}$ at the $\underline{P} = \underline{.01}$ level.

and second series of BCG immunized animals, 698 ± 117.13 g and 624 ± 94.87 g in the irradiated vaccine groups while the controls weighed 617 ± 63.90 g. Death from generalized and advanced tuberculosis occurred in 8 (44%) control animals between 65 and 102 days after the virulent inoculation, in 3 and 4 animals in the 2 series of BCG immunized animals (15.9%) and in 5 and 6 animals in the series of irradiated vaccine animals (31.4%). In order to assess the tissue resistance against the challenge inoculation in the variously immunized animals before progression of the infection obliterated it, it was decided to kill the surviving animals after approximately half of the non-immunized control animals had died spontaneously with generalized tuberculosis. This was done between 102 and 105 days after the challenge virulent inoculation.

The same autopsy procedure was adhered to as on previous occasions(9) and the completed data were tested for significant differences by Fisher's method(10) for comparison of 2 means. Autopsies revealed that all the animals having received 3 intraperitoneal inoculations with H37Rv and 199RB irradiated vaccines presented an advanced form of dry tuberculous peritonitis with little or no fluid in the peritoneal cavity, but a dense plastic exudate gluing the intestines together by firm adhesions which separated with difficulty. These findings were in striking contrast to the nearly complete absence of fibrous adhesions in the peritoneal cavity of the BCG immunized animals in which the omentum was regularly thickened and contracted into a sausage-like band containing numerous caseous nodules from which typical BCG virulent organisms were cultured on Löwenstein's medium. The gross pathological picture of the non-immunized control animals showed localized tuberculous lesions in only 2 animals (11%) while the remaining 16 animals presented various degrees of advanced and fulminating tuberculosis. We were greatly surprised to find 13 and 12 animals in the 2 series of BCG immunized groups (56.8%) completely protected against the spread of the challenge inoculation, and 4 in each group (18.2%) presenting only localized tuberculous lesions and the remaining 5 and 6 animals (25%) showing advanced and generalized tuberculous lesions. Table I shows that neither group of animals immunized with irradiated vaccine showed an animal completely protected against the challenge inoculation, that 9 and 10 animals (54.3%) presented localized tuberculous lesions and the remaining 8 animals in each group (45.7%) showing advanced and generalized tuberculosis.

The quantitative data on enlarged regional lymph glands, the pooled total 17 sets of routinely dissected lymph nodes, and on the spleen, liver and lungs from animals immunized with 2 types of irradiated vaccine and BCG vaccine, together with those from the control animals, are given in Table I. These data bear out the gross pathological findings mentioned above, namely that irradiated killed tuberculosis vaccine is surely capable of producing specific resistance against a challenge virulent tuberculous infection. But in comparison with either series of BCG immunized animals, the protective value of living BCG vaccine is significantly greater than that of killed irradiated tuberculosis vaccine. In view of the incomplete immunity produced in animals and in man with the relatively avirulent BCG living organisms, our study suggests that it would be premature to substitute for BCG a vaccine consisting of killed tubercle bacilli.

Summary. Living BCG vaccine has shown superior antigenic potency to killed irradiated tuberculosis vaccines by producing significantly greater tuberculin hypersensitiveness and specific resistance against a challenge virulent inoculation in guinea pigs.

1. Myers, J. A., *Am. Rev. Tuberc.*, 1948, v57, 107; *J.A.M.A.*, 1951, v146, 1492.

2. Hilleboe, H. E., *Am. Rev. Tuberc.*, 1947, v55, 294.

3. Statement on BCG, American Trudeau Society, *Am. Rev. Tuberc.*, 1949, v60, 681.

4. Calmette, A., L'infection bacillaire et la Tuberculose. Masson et Cie, Paris, 1936, pp. 838-981; Corper, H. J., *J. Lab. and Clin. Med.*, 1946, v31, 346; Irvine, K. N., B.C.G. Vaccination in Theory and Practice, Blackwell, Oxford, 1949, pp. 49-77; Opie, E. L., Flahiff, E. W., and Smith, H. H., *Am. J. Hyg.*, 1939, v29, 155.

5. Olson, B. J., Habel, K., and Piggott, W. R., Publ. Health Rep., 1947, v62, 293.

6. Sarber, R. W., Nungester, W. J., and Stimpert, F. D., *Am. Rev. Tuberc.*, 1950, v62, 418.

7. Milzer, A., Levinson, S. O., and Lewis, M. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 733.

8. Birkhaug, K., *Am. Rev. Tuberc.*, 1949, v59, 567.

9. Birkhaug, K., *Acta med. Scand.*, 1944, v117, 274; *Am. Rev. Tuberc.*, 1951, v63, 85.

10. Fisher, R. A., Statistical Methods for Research Workers, ed. 6. Oliver & Boyd, London, 1936, pp. 128-133 and Table IV.

Received December 5, 1951. P.S.E.B.M., 1952, v79.

Ionization and Distribution Between Aqueous and Non-Aqueous Solvents of Dyes in Relation to Gastric Secretion.* (19390)

SIEGFRIED WOISLAWSKI. (Introduced by F. E. Ray.)

From the Cancer Research Laboratory, University of Florida, Gainesville.

Recently(1) a formula was devised in this laboratory that correlated the pK_B of certain basic dyes with their tendency to be secreted by the stomach. While successful in leading to the discovery of new compounds that were secreted in substantial amounts, certain discrepancies were evident(2). In the new series only compounds with a pK_B over 10 were secreted while in the original series the optimum was 6.5 to 9. These discrepancies might be caused by the fact that highly purified dyes were used in the original(1) pK_B determinations while Ingraham and Visscher

---

* Supported by a Grant from the Anna Fuller Fund.

TABLE I. Comparison of pK$_B$ of Pure and Commercial Dyes.

Compound	C.I. No.	Stomach* / Blood	pK$_B$		Absorption maxima (mμ)
			Pure	Com'l	
Nile blue sulfate	913	.8	11.6	10.9	640
Acridine red	740	8.4	10.9	5.9	555
Rhodamine B	749	6.1	10.8	10.3	555
Brilliant cresyl blue	877	11.3	10.8	9.4	625
Methylene green	924	13.6	10.8	9.9	620
Neutral violet	826	27.2	10.8	7.4	535
Methylene blue	922	15.6	10.2	—	660
Bismark brown	331	26.5	9	9.2	440
Chrysoidin Y	20	75	8.7	9	455
Safranin O	841	27.3	7.6	—	520
Neutral red	825	26.4	7.5	7.3	530
Thionin	920	36.8	7.1	7.2	600
Toluidine blue	925	36.4	6.5	6.1	625
Pyronine B	741	5.7	6.3	7.1	550

* Ref. 3.

(3) in their determination of the stomach: blood ratios used commercial products. Although there is no certainty that the present commercial dyes are identical with those used by the authors mentioned, it seemed of interest to repeat the pK$_B$ determinations on the commercial products. The weakness of the pK$_B$ determination of polybasic compounds is that it is a stepwise process. It was thought that the distribution between an acid buffer and benzene might be a better measure of total effective basicity and would correspond more closely with the stomach:blood ratios.

Methods. A. Basic ionization constants were determined by potentiometric titration employing the Beckman G, pH meter with glass electrode 1190T and calomel electrode 1170. Beckman electrode 1190E was used when the pH rose above 10. The electrodes were checked in standard buffers before and after each determination. The room temperature was maintained at 25 ± 1°. From 0.1200 to 0.2500 g of dye was dissolved in 300 ml of water and titrated with 0.048N sodium hydroxide. Values were taken from the curves in the usual manner. *B.* Equal volumes (5 ml) of buffer, $NaHSO_4/Na_2SO_4$, pH 2, or KH_2PO_4/Na_2HPO_4, pH 6.73, and benzene were shaken together with the dye until equilibrium was achieved. The two phases were separated and the concentration measured in a Beckman Model DU spectophotometer and compared with a standard. Dyes that were insoluble in the buffer were first dissolved in water and then diluted with buffer solution. By using samples of 1 to 10 μg per ml Beer's Law was valid. Because of the dilute solutions used, Dalton's Law applies and inorganic impurities that may be present do not effect the results. The absorption maxima are listed in the last column of Table I.

Results. A. Basic ionization constants. Most of the pK$_B$ determinations on the impure dyes agree rather well with those reported for the purified substances. In Table I the compounds are arranged in the order of decreasing pK$_B$ (increasing basicity) of the pure compounds. The titration curves of the commercial dyes did not rise as sharply nor were the plateaus as well defined. In fact 2 compounds gave such obscure curves that no definite values could be assigned. Acridine Red seems to be one compound that shows widely differing values: 10.9 for the pure and 5.9 for the impure. Its transposition to the bottom of the Table would serve to place it just as satisfactorily on the descending end of the curve. Neutral Violet on the new basis would also fall into a more favorable position next to Safranin O which has a similar stomach:blood ratio. Pyronine B, however, with a stomach:blood ratio of 5.7 would now be located between Thionin and Toluidine Blue, both of which have stomach:blood ratios of about 36. Thus the position of most compounds is unaffected; 2 compounds are better placed; while one is misplaced by the data

TABLE II. Distribution Ratios (Commercial Dyes).

Compound	C.I. No.	pH buffer	Buffer / Benzene	Stomach† / Blood
Nile blue sulfate	913	2	2.7	.8
Chrysoidin Y	20	2	4.3	75
Bismark brown	331	2	5.7	26.5
Neutral violet	826	2	‡	27.2
Neutral red	825	2	”	26.4
Toluidine blue	925	2	”	36.4
Nile blue sulfate	913	6.73*	§	.8
Bismark brown	331	6.73	”	26.5
Chrysoidin Y	20	6.73	”	75
Neutral violet	826	6.73	.68	27.2
Pyronine B	741	6.73	1.96	5.7
Methylene green	924	6.73	2.5	13.6
Brilliant cresyl blue	877	6.73	2.7	11.3
Toluidine blue	925	6.73	2.9	36.4
Safranin O	841	6.73	5.6	27.3
Thionin	920	6.73	11.5	36.8
Methylene blue	922	6.73	‡	15.6

* Neutral Red, Acridine Red and Rhodamine B became colorless when shaken with benzene buffer at pH 6.73. † Ref. 3. ‡ 100% in buffer. § 100% in benzene.

on commercial dyes. The stomach:blood ratio for Chrysoidin Y is decidedly out of line with all other values and must be presumed to be in error. The new dyes of the fluorene series (2) were found not to be secreted by the stomach of the rat at a pK_B as low as 8.5. It is obvious that the results on the commercial dyes do not throw any light on this. In fact, by changing the value of Neutral Violet from 10.8 to 7.4 it definitely takes the one good analogous value out of consideration.

B. Distribution ratios. In Table II the compounds are arranged in the order of decreasing concentration in the benzene layer (order of increasing basicity). Two different buffers were used, pH 2 and 6.73. Three of the compounds, Acridine Red, Rhodamine B and Neutral Red became colorless or yellow when shaken with the benzene-buffer at pH 6.73. On evaporation of the benzene the red color returned. Probably the free bases are colorless and the compounds are thus unsatisfactory for this type of investigation. Nile Blue becomes red because of oxazone formation. It too, is unsuitable. In column 5 of Table II, the stomach:blood ratios are those given by Ingraham and Visscher(3). Column 4 gives the values for the buffer:benzene. In considering the first series measured at pH 2 the distribution ratios parallel pK_B values for the commercial dyes. The pK_B determination for pure Neutral Violet is again less satisfactory than that of the commercial dye. The stomach:blood ratios also fall fairly well on a plateau for compounds with a buffer:benzene ratio of 5.7 to infinity. The stomach:blood ratio for Chrysoidin seems to be entirely out of line with all other values and can only be accepted with reservation. Experiments in this laboratory, however, show that its secretion is at least as great as that of Neutral Violet.

Since half of the dyes went 100% into the buffer at pH 2, it was decided to increase the pH in order to secure a better evaluation. The results are shown for pH 6.73 in the lower section of Table II.

Here the 3 compounds that gave finite values for the distribution ratio at pH 2 were completely in the benzene layer but only one, Methylene Blue, was entirely in the buffer. A careful inspection of the data at pH 6.73 reveals no relation between the distribution ratios and either the stomach:blood ratios or the pK_B values of pure or commercial dyes. A pH of 6.73 is thus definitely unsuitable for this type of investigation. A value intermediate, say pH 4, would result, however, in other compounds besides Methylene Blue being entirely in the buffer layer.

Summary. In a study of the relation of physical chemical properties of dyes to the extent of their secretion by the stomach, new determinations of the pK_B values and absorption maxima were made. A somewhat better correlation of pK_B and stomach: blood ratios was obtained by using commercial instead of pure samples of dyes. The distribution of the dyes between benzene and aqueous buffers of pH 2 and pH 6.73 was measured but no satisfactory correlation found. The method is limited by the tendency of compounds to dissolve 100% in either the buffer or the benzene.

1. Ray, F. E., and Jung, M. L., *Brit. J. Cancer*, 1951, v5, 358.

2. Ray, F. E., and Peters, J. H., *Brit. J. Cancer*, 1951, v5, 364.

3. Ingraham, R. C., and Visscher, M. B., *J. Gen. Physiol.*, 1935, v18, 695.

Received January 3, 1952. P.S.E.B.M., 1952, v79.

Inactivation of 11-Dehydro-17-Hydroxycorticosterone by Tissue Slices.* (19391)

JEAN LOUCHART† AND JOSEPH W. JAILER.‡

From the Departments of Medicine and Obstetrics & Gynecology, College of Physicians and Surgeons, Columbia University and Presbyterian Hospital, New York City.

Numerous investigations carried out during the past several years have clearly demonstrated that the liver is the organ most concerned with the inactivation of estrogens, testosterone and desoxycorticosterone(1-3). However, when 11-dehydro-17-hydroxycorticosterone (cortisone) became available for study, it became apparent that degradation of such 11-oxygenated steroids might differ from that of estradiol, testosterone and desoxycorticosterone. The latter steroids are practically ineffective when given orally, whereas cortisone§ is approximately 80% as effective orally as parenterally. The following data however, strongly suggest that the 11-oxygenated steroids are rapidly inactivated by the body. A very small percentage of administered cortisone can be recovered from the urine of the patients. The finding of relatively high concentrations of 11-oxygenated steroids in the adrenal vein blood, and of very little in peripheral blood(4,5) is also suggestive of inactivation. deAndino, Fontan, and Pashkis (6) showed that intrasplenic injection of cortisone was ineffective in decreasing thymic weight unless huge doses were administered, thus implicating the liver in the inactivation of this steroid.

The purpose of this study was to determine which organs of the body might inactivate cortisone *in vitro*. In addition, experiments were devised to ascertain whether the inactivation mechanism could be modified.

Methods and materials. Rat tissue slices taken from normal male rats immediately after decapitation were used for studies of cortisone inactivation. Liver, kidney, spleen, muscle, and brain were sliced by hand; 300 to 350 mg of tissue were incubated in 5 ml of Krebs-Ringer solution (pH 7.4) with 100 to 250 μg of cortisone for 3 hours at 37°C. After incubation, the tissue slices were homogenized in a Ten Broeck homogenizer, kept at 4°C overnight and the whole homogenate bioassayed on the following morning as described below. Two types of control were used: 1) tissue slices which had been boiled previously for 10 minutes; 2) non-incubated controls to which the cortisone was added immediately before bioassay. This was performed to exclude the possibility of adsorption of the steroid to the tissue protein. Cortisone, in the form of the free alcohol, was prepared in 95% ethanol in the concentration of 1 mg/ml and 0.1-0.25 ml of this standard solution were

---

* Aided by grants from the American Cancer Society as recommended by the National Research Council, Committee on Growth and the Ciba Pharmaceutical Co.

† Squibb Fellow in Endocrinology.

‡ Damon Runyon Senior Clinical Research Fellow.

§ Kindly supplied by Merck, Inc.

added to the Krebs solution. The amount of cortisone remaining after incubation was determined by bioassay according to the method of Venning, Kazmin, and Bell(7) with certain modifications. Male mice (Rockland Farm strain) weighing 20 to 25 g and adrenalectomized 3 days previously, were used. They were fasted for 12 hours, and on the morning of the assay each mouse received 0.25 ml of the homogenate to be assayed plus 0.25 ml of 5% glucose in physiological saline at 2-hour intervals for 4 injections. Approximately equal numbers of control and experimental animals were used. One hour after the last injection, the animals were weighed and killed by severing the vertebrae. The livers were removed within 30 seconds and glycogen determined by the method of Nelson(8). Dose response curves for mouse liver glycogen were established at levels of 10, 25, 50, 75 μg of cortisone. With each experiment, cortisone was incubated with boiled tissue slices and the amount of cortisone recovered in this manner was used as the index for calculating the loss of activity of the cortisone incubated with the living slices. Occasionally, the recovery appeared to be greater than 100%; this indicated only that in the particular experiments the glycogen deposition was greater than the mean value obtained on the standard curve. Liver glycogen analyses, after the administration of cortisone and cortisone with boiled tissue slices were performed on 586 mice with an accuracy of ±20%.

Results. I. Tissue slices. The results are presented in Tables I and II. Slices of liver, kidney, and spleen inactivate cortisone to approximately the same extent, under the conditions of this experiment. The average percentage of degradation by liver slices was 70%; kidney, 61%; spleen, 70%. Similar results were obtained using Upjohn's Aqueous Adrenocortical Extract (Table I). On the other hand, incubation with muscle, brain, and blood serum caused negligible inactivation of the cortisone (Table II).

TABLE I.

No. of assay animals (control + exp.)	Dose of cortisone incubated (per ml) μg	Recovery, μg		Inactivation, %
		Controls	Fresh tissue	
Inactivation of cortisone by liver slices				
15	20	25	14	44
12	20	15	7	53
12	20	15	6.5	57
12	20	24	4	83
7	20	25	0	100
9	20	15	0	100
10	.5 ml (Ad. cort. extr.)	50	14	72
Inactivation of cortisone by spleen slices				
11	20	26	3.5	87
11	20	40	5.5	86
14	42	52	16	69
25	50	63	13	79
16	30	42	30	29
16	50	58	28	52
16	50	100	34	66
Inactivation of cortisone by kidney slices				
12	20	20	4	80
14	20	20	5	75
8	20	17	9	46
12	20	16	8	50
10	42	32	14	56
11	.5 ml (Ad. cort. extr.)	41	18	56

TABLE II. Inactivation of Cortisone by Muscle, Brain and Blood Serum.

No. of assay animals (control + exp.)	Dose of cortisone incubated (per ml) μg	Recovery, μg		Inactivation, %
		Controls	Fresh tissue	
Muscle				
9	20	17	15	17
10	20	11	18	0
12	42	23	14	22
11	42	51	45	17
14	50	17	20	0
Brain				
13	20	20	37	0
12	42	70	38	46
14	42	15	20	0
13	50	54	49	9
Blood serum				
9	20	24	20	16
13	20	20	17	15
11	20	19	22	0
12	42	48	42	12

II. Tissue homogenates. DeMeio, Rakoff, Cantarow, and Pashkis(9) and Coppedge, Segaloff, Sarett, and Altschul(10) demonstrated that while liver slices were capable of inactivating estradiol, homogenized liver lost most of this ability. They concluded that cellular integrity must be intact for the ef-

TABLE III. Inactivation of Cortisone by Homogenized Liver Slices.

No. of assay animals (control + exp.)	Dose of cortisone incubated (per ml), μg	Recovery			Inactivation	
		Controls	Homogenized tissue, μg	Fresh tissue	Homogenized, %	Fresh, %
7	42	32	35		0	
9	42	43	33		23	
14	50	70	40	24	43	66
13	50	41	24	12	41	71
11	50		29	10	42	80

ficient working of the enzyme system. The addition of certain cofactors (nicotinamide or diphosphopyridine nucleotide) to liver homogenates restored the inactivation system to normal. Similarly, incubation of homogenized liver instead of liver slices resulted in a definite decrease from 70% to 25% in the percentage of inactivation of cortisone, under these conditions (Table III).

III. Influence of other steroids. Pregnenolone, testosterone propionate, desoxycorticosterone, progesterone, and cortisone were administered to normal rats for a period of 10 days, and liver slices from these animals incubated with cortisone as above. This was done in an attempt to ascertain whether the enzyme system responsible for the inactivation of cortisone could be saturated by other steroids. None of these steroids exerted an inhibitory effect on the inactivation of cortisone. Under these conditions, the average percentage of inactivation was 71% as compared to 70% for the controls.

IV. Influence of stress. Liver slices from adrenalectomized rats, maintained for a week on 1% NaCl as drinking water, were also tested. No significant difference between the inactivation capacity of liver tissue from such animals as compared to normal ones was apparent. Normal rats were exposed to cold (4°C) for 24 hours and their livers tested. This procedure also produced no change in the percentage of inactivation.

V. Effect of incubation with glucuronidase. Recent investigations have demonstrated that the great bulk of urinary cortico-steroids are conjugated as glucuronides(11,12). Thus in order to break any possible glucuronide linkage which might be responsible for cortisone inactivation, 3,000 units of animal glucuronidase were added to each 5 ml homogenate after the usual 3 hours of incubation. They were then incubated again for 24 hours at 37°C. This procedure did not increase the recovery of cortisone. The percentage of inactivation after glucuronidase incubation was 69% while the values for the controls averaged 71%.

Discussion. Cortisone can be inactivated by kidney and spleen as well as liver slices. This is in direct contrast with the estrogens, androgens and desoxycorticosterone which appear to be degraded primarily by the liver. Although nothing is known of the enzyme system involved, it is clearly heat-labile, and certain cofactors are necessary for its efficient operation. This is evidenced by the fact that destruction of the integrity of the cells by homogenization markedly reduces the degree of inactivation of cortisone. Similar results have been obtained with other steroids(9,10).

Sayers(13) has postulated that under conditions of stress, there is greater peripheral utilization of adrenal steroids. It was thought to be of interest to determine whether under conditions of stress, tissues inactivated cortisone to a greater extent. Liver slices of rats exposed to cold exhibited the same degree of cortisone inactivation as normal or even adrenalectomized animals. The pre-administration of pregnenolone, testosterone, progesterone, desoxycorticosterone, and cortisone in an attempt to saturate the inactivation system was also ineffective.

Summary. 1. Slices of liver, spleen, and kidney incubated in Krebs solution for three hours with added cortisone caused approximately a 70% loss of cortisone activity. Blood serum and brain or muscle slices were inactive. Tissue homogenates were much less effective than slices. Incubation of the tissue-cortisone

mixture with the enzyme, glucuronidase did not alter the experimental findings. 2. There was no detectable difference in the capacity of tissues of stressed or adrenalectomized animals, as compared to tissues from normal rats, to inactivate cortisone. The inactivation mechanism could not be altered by the preadministration of progesterone, pregnenolone, testosterone, desoxycorticosterone or cortisone.

We wish to express our appreciation to Miss Beatrice Silides for her technical assistance.

1. Jailer, J. W., *J. Clin. Endocrinol.*, 1949, v9, 557.
2. Samuels, L. T., *Recent Progress in Hormone Research*, 1949, v4, 65.
3. Heard, R. D. H., Saffran, J. C., *Recent Progress in Hormone Research*, 1949, v4, 25.
4. Vogt, M., *J. Physiol.*, 1943, v102, 341.
5. Nelson, D. H., Reich, H., and Samuels, L. T., *Science*, 1950, v111, 578.
6. deAndino, A. M., Fontan, J. L. R., and Pashkis, K. E., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 700.
7. Venning, E. H., Kazmin, V. E., and Bell, J. C., *Endocrinology*, 1946, v38, 79.
8. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.
9. DeMeio, R. H., Rakoff, A. E., Cantarow, A., and Pashkis, K. E., *Endocrinology*, 1948, v43, 97.
10. Coppedge, R. L., Segaloff, A., Sarett, H. P., and Altschul, A. M., *J. Biol. Chem.*, 1948, v173, 431.
11. Kinsella, R. A., Jr., Doisy, R. J., and Glick, J. H., Jr., *Fed. Proc.*, 1950, v9, 190.
12. Corcoran, A. C., Page, I. H., and Dustan, H. P., *J. Lab. and Clin. Med.*, 1950, v36, 297.
13. Sayers, G., *Physiol. Rev.*, 1950, v30, 270.

Received January 10, 1952. P.S.E.B.M., 1952, v79.

Relation of Stage of Cycle and Source of Luteinizing Hormone to Superovulation in Dairy Cattle.* (19392)

E. L. WILLETT, W. H. MCSHAN, AND ROLAND K. MEYER.

From the American Foundation for the Study of Genetics, Madison, and the Department of Zoology, University of Wisconsin.

In research to develop practical technics for egg transplantation in dairy cattle, the first problem is that of superovulation. This problem has already been investigated by various workers(2-10). When the research reported here was initiated, some workers(2,3,10) had injected cows or heifers with gonadotrophins during the luteal phase of the cycle without expressing the corpus luteum, and the evidence indicated, although not conclusively, that cows superovulated and bred under these conditions produced infertile eggs. Rowson(8) later reported that eggs, shed by cows which had been bred and which had a functioning corpus luteum, were infertile. Various sources of gonadotrophins have been employed by different workers, including unfractionated sheep pituitary gonadotrophin (USG) and human chorionic gonadotrophin (HCG) for intravenous injections to rupture the follicles. No one has compared these two gonadotrophins quantitatively.

This paper reports the results of two series of investigations with heifers and cows to study primarily a) the effect of the stage of the cycle upon ovarian response to gonadotrophins and b) the quantitative difference in response to USG and HCG.

Experimental. Study of stage of cycle, dosages of FSH and two sources of LH. The first series consisted of a factorial experiment. It was designed to study follicular development and number and fertility of ova as affected by: a) initiation of treatment on the fourth day of the cycle (luteal) versus sixteenth day (follicular), b) 3 levels of follicle stimulating hormone (FSH), namely 20, 30 and 40 g-equivalents (g.e.) of desiccated sheep pituitaries, and c) one g.e. of USG versus 2500 I.U. of HCG (Choriogonin) for the intravenous injection. In this experiment 12

---

[*] A brief report of part of this work was given at 40th Annual Meeting of American Society of Animal Production, Nov. 26, 1948(1). The authors are indebted to Dr. J. L. Davidson, The Upjohn Co., for generous supplies of Gonadogen and to Dr. R. S. Justice, Lakeside Laboratories, for liberal quantities of Choriogonin.

yearling Holstein heifers were divided into two equal groups, one the "luteal" and the other the "follicular" group. Two of the animals in each group received 20, two 30 and two 40 g.e. of FSH. One of each of these pairs received USG and the other HCG for the intravenous injection. The heifers were allotted in a random manner. The treatment consisted of: a) FSH administered in equal portions subcutaneously back of the shoulders daily for 5 days, b) intravenous injection on the 6th day, and c) insemination on the 6th and 7th days. The FSH was from one batch prepared by slight modification of the method of McShan and Meyer(11). A total of 1½ g.e. given subcutaneously twice daily for 10 days to 30-day-old Sprague-Dawley hypophysectomized rats produced ovaries containing only follicles and averaging 130 mg; 3 g.e. produced similar ovaries averaging 192 mg. In another test with the method of McShan and Meyer, 1 g.e. (6.4 mg of lyophilized extract) produced ovaries averaging 54 mg in 21-day-old, intact rats. The batch of unfractionated sheep gonadotrophin was prepared by the method of McShan and Meyer (12). One g.e. of the lyophilized extract weighed 80.1 mg. One-tenth g.e. (8 mg) produced ovaries averaging 85 mg when assayed as was the FSH with intact rats. The heifers were inseminated with semen from bulls of high fertility. Immediately after slaughter the reproductive organs were removed, and the horns and oviducts were flushed with physiological saline solution. Recovered eggs were examined in hanging drop slides at a magnification of 440X. The number of ova produced, as indicated by number of ovulation points or corpora lutea, was the main criterion for measuring ovarian response. The ovaries were weighed, unruptured follicles counted, and their diameters measured with calipers. In an attempt to recover eggs from the live animal, the uteri of a few heifers were irrigated an hour or two prior to slaughter on the fifth day following the intravenous injection. No eggs were recovered, and since it appeared that the number of ova recovered was reduced, this practice was discontinued. Furthermore, after killing 2 "luteal" heifers on the 5th day, it was found that eggs could not be recovered from the uterine washings due to pyometra. The slaughtering of all heifers was subsequently done on the 3rd day at which time most of the eggs are in the oviducts.

The responses to the various treatments were extremely variable as has been reported by other workers. A summary of results is presented in Table I. Almost twice as many corpora were produced during the follicular as during the luteal phase. Folley and Malpress(5), on the other hand, observed no difference in ovarian response between the follicular and the luteal phase with the corpus luteum intact, while Rowson(8) had considerably more ovulations during the luteal phase. These discrepancies may possibly be explained by differences in treatments or criteria. None of the 34 eggs recovered in this experiment from luteal heifers showed evidence of having been fertilized, whereas 74% of the 43 from follicular heifers were fertilized. The eggs from the follicular group had blastomeres of normal uniformity in size, and spermatozoa were present in the zona pellucida. The evidence of infertility during the luteal phase is in agreement with findings of other workers (2,3,8,10). In light of all data now available, there is no doubt that bovine females, ovulated by gonadotrophins in the presence of the corpus luteum, produce infertile eggs as shown by lack of fertilization on breeding. Studies by Murphree *et al.*(13) indicate that interference with spermatozoan transport through the cervix accounts for much of the infertility in luteal phase rabbits. With the bovine, on the other hand, it appears as though other factors may be responsible. Most of the luteal heifers in the experiment described in this paper were inseminated in the uterus. Furthermore, Beschlebnov(14) and VanDemark and Moeller(15) have found that sperm reached the ovaries when cows were inseminated while not in heat.

All the heifers in the luteal group had pyometra. Since this observation was originally reported(1), a comparable condition has been reported in swine(16) and rabbits(13). It is improbable that substances in the semen diluter (egg yolk-citrate) used for breeding the heifers were responsible, for one heifer was inseminated in the cervix with undiluted se-

TABLE I. Summarization of Comparisons Between Various Treatments in Factorial Experiment. Figures are heifer averages.

Treatment	Total No. heifers	Wt of ovaries, g	No. Unruptured follicles >4 mm diam.	No. corpora lutea	No. heifers yielding eggs	Eggs: No. ovulated	Eggs: No. recovered	Eggs: % cleaved
Luteal	6	36	20	12.7	5	14.4	6.8	0
Follicular	6	53	28.2	21.8	5	23.4	8.6	74
20 g.e. FSH	4	34	19	11.8	2	14.5	8	6
30 ״ ״	4	50	29.8	22.2	4	22.2	11.2	62
40 ״ ״	4	48	23.5	17.8	4	17.8	4	19
USG	6	42	30.5	23	6	23	9.7	6
HCG	6	47	17.7	11.5	4	12.8	4.8	26

men because of inability to penetrate the cervix with the insemination tube. All other heifers were inseminated in the uterus with semen in yolk-citrate diluent. Whether changes during the cycle in regard to uterine sensitivity to foreign substances or to defenses against bacteria introduced at insemination or whether other factors produced this pyometra merit investigation. An answer to this problem might shed light on the cause of spontaneously occurring pyometra in cows(17).

The lowest dose of FSH gave the lowest response; there was not much difference between the two higher dosages. USG injected intravenously produced twice as many corpora as HCG. The data from this experiment were tested by analysis of variance. Due to the small number of heifers and the great variability, there were no significant differences between any of the treatment means. Neither were there any significant interactions between any two of the treatments.

Further study of two sources of LH. The data from the above experiment were considered adequate in regard to stage of cycle and dosage levels of FSH. It was concluded, however, that more information was needed as to the relative number of corpora produced with USG and HCG. Consequently, a second series of animals was used to obtain more data for this comparison. Heifers or cows were superovulated by giving a single subcutaneous injection of gonadotrophin from pregnant mare serum (PMS) on the 15th or 16th day of the cycle and an intravenous injection of USG or HCG five days later. Dosages given were 2,000, 3,000 or 5,000 I.U. of PMS, 2,500 or 5,000 I.U. of HCG and 1 g.e. of USG. Animals were paired as to size, breed and parity. The 2 animals in each pair received the same dose of the same batch of PMS on the same day of the cycle, but one received USG and the other HCG for the intravenous injection. There were 6 pairs of Holsteins and one of Guernseys. The 5 pairs of heifers were slaughtered, and the ovaries studied in the laboratory. Animals in 2 of the pairs were mature cows, and the number of corpora lutea in their ovaries was counted *in vivo* by making an incision through the dorsal wall of the vagina and the peritoneum of the recto-vaginal pouch, drawing the ovaries into the vagina, and examining them with a speculum.

The data for individual heifers are presented in Table II. The data from this series were combined with those from series I, for the animals in both series had been paired similarly.

A summary of the data from the 2 series and of the combined data is given in Table III. Results from the 2 series were similar in that a greater number of corpora was obtained with USG than with HCG. When the combined data were analyzed by analysis of variance, it was found that the over-all means were almost highly significantly different (probability slightly greater than 0.01). There was no significant interaction between the subcutaneous and intravenous treatments. The evidence that a larger number of corpora can be obtained with USG than with HCG is,

TABLE II. Ovarian Response and Infertile and Fertile Eggs Recovered from Individual Heifers and Cows in Series II Receiving PMS and Two Sources of LH.

No. of pair	No. of heifer	Dosages: PMS, I.U. × 1000	Dosages: USG, g.e.	Dosages: HCG, I.U. × 1000	Interval intrav. to autopsy, days	Wt of ovaries, g	No. unruptured follicles: 5-25 mm	No. unruptured follicles: >25 mm	No. corpora lutea	Eggs: No. recovered	Eggs: Cleaved No.	Eggs: Cleaved %
I	22	5	1		5	61	14	0	27	16	14	87
II	28	3	1		7	205	16	5	33	4	1	25
III	33	2	1		5	25	11	0	10	5	4	80
IV	54	3	1		—	—	—	—	39	—	—	—
V	57	2	1		—	—	—	—	6	—	—	—
VI	67	2	1		8	18	1	0	5	1	0	0
VII	68	2	1		6	46	4	0	8	5	5	100
Avg						71	9.2	1	18.3	6.2	4.8	77
I	21	5		2.5	5	33	6	0	14	8	5	62
II	27	3		2.5	4	29	7	0	4	1	1	100
III	32	2		5	5	52	4	0	12	6	6	100
IV	55	3		5	—	—	—	—	2	—	—	—
V	56	2		5	—	—	—	—	2	—	—	—
VI	65	2		5	8	15	5	0	1	0	0	—
VII	60	2		2.5	4	12	5	0	1	0	0	—
Avg						28	5.4	0	5.1	3	2.4	80

therefore, quite conclusive. Umbaugh(9) also presented data indicating that such differences might occur, although other variables were involved in the comparison. It would appear, on the basis of the limited data for individual heifers (Table II), that the dosage of HCG was not a factor causing this difference in our experiments, for the relationship of the response with HCG to that with USG treatment was apparently the same whether 2,500 or 5,000 I.U. of HCG was given. Probably USG produces more corpora when given intravenously than HCG because it contains considerably more FSH. In this connection, it is of interest that, in both experiments, USG also produced more unruptured follicles than did HCG. This difference in number of follicles was not significant, however.

A comparison of the data from series I with series II gives some indication of the relative merits of sheep FSH and PMS. Injections with PMS-USG have frequently resulted in large ovaries with follicles greater than 25 mm in diameter, which might be classed as cystic. One heifer in series II, No. 28, had such follicles. In cases where the animals were not slaughtered, these large follicles were later resorbed. Dowling(4), and Folley and Malpress(5) have observed such abnormal responses when injecting PMS and the latter have also observed resorption. There is a possibility that PMS-USG overstimulates the ovaries of some animals causing a rapid thickening of the follicle wall and thus preventing ovulation(18). The presence of some LH in PMS may aggravate this condition.

The animals injected with PMS and HCG had fewer corpora than those receiving PMS and USG but did not have extremely large ovaries or follicles. Of 15 eggs recovered from this group, 80% had apparently cleaved normally. One animal in this group received the highest dose of PMS (5,000 I.U.) and also 2,500 I.U. HCG. She produced 14 corpora. Eight eggs were recovered and 5 had cleaved. This combination of PMS and HCG merits additional investigation in reference to its possibilities of producing superovulation.

The 5 pairs of animals in series II which were slaughtered and whose eggs were studied

TABLE III. Number of Unruptured Follicles and Corpora Lutea in Ovaries of Heifers and Cows in Series I and II Receiving USG or HCG in the Intravenous Injection. Figures are avg.

Series	Subcut. inj.	No. animal pairs' follicles studied	No. unruptured follicles >4 mm diam.		No. animal pairs' corpora studied	No. Corpora lutea	
			USG	HCG		USG	HCG
I	Sheep FSH	6	30.5	17.7	6	23	11.5
II	PMS	5	10.2	5.4	7	18.3	5.1
	Totals and avg	11	21.3	12.1	13	20.5	8.1

were killed on the 5th to 8th day following the intravenous injection. All had been inseminated at the time of the intravenous injection and the day following. Of the 31 eggs recovered from the PMS-USG treated animals, 77% had apparently cleaved normally. The one animal in this series (No. 28) which had extremely large ovaries had a total of 33 corpora. She was killed on the 7th day following the intravenous injection. Three of 4 eggs recovered had not cleaved. The fourth apparently had, but the blastomeres showed evidence of degeneration. Dowling(4) has found that, when animals were injected during the follicular stage of the cycle with PMS and when abnormal ovaries were produced, a small percentage of the ova were fertilized but abnormal in appearance.

The question has frequently been raised as to whether fertilized superovulated bovine eggs are capable of normal development. The American Foundation for the Study of Genetics has data indicating that at least some of them are. As of this writing, one living calf and 2 pregnancies (114 days and 150 days) confirmed by palpation have been produced from 5 transplantations. These transplantations have been performed with methods similar to those described in the report of the birth of the calf(19), including superovulation of the donor with sheep FSH and USG during the follicular stage of the cycle.

Summary. 1. Two series of studies involving a total of 26 heifers and cows are reported. 2. Five heifers superovulated during the luteal phase of the estrual cycle produced 34 eggs none of which were fertilized. Five heifers treated during the follicular phase produced 43 eggs of which 74% had cleaved. All 6 heifers treated during the luteal phase, but none of the 6 follicular heifers, had pyometra. 3. Thirty and 40 g.e. of sheep FSH gave responses about equal but considerably greater than a 20 g.e. dose. 4. USG produced approximately twice as many corpora as HCG in the intravenous injection when they were used in combination with either sheep pituitary FSH or PMS. 5. A combination of PMS and HCG may produce satisfactory superovulation and should be studied further.

---

1. Willett, E. L., McShan, W. H., and Meyer, R. K., *J. Anim. Sci.*, 1948, v7, 545.

2. Casida, L. E., Meyer, R. K., McShan, W. H., and Wisnicky, W., *Am. J. Vet. Res.*, 1943, v4, 76.

3. Casida, L. E., Nalbandov, A., McShan, W. H., Meyer, R. K., and Wisnicky, W., *Proc. Am. Soc. Anim. Prod.*, 1940, p302.

4. Dowling, D. F., *J. Agr. Sci.*, 1949, v39, 374.

5. Folley, S. J., and Malpress, F. H., *Proc. Roy. Soc., B.* 1944, v132, 164.

6. Hammond, J., Jr., *J. Agr. Sci.*, 1949, v39, 222.

7. Hammond, J., Jr., and Bhattacharya, P., *J. Agr. Sci.*, 1944, v34, 1.

8. Rowson, L. E., *J. Endocrinology*, 1951, v7, 260.

9. Umbaugh, R. E., *Am. J. Vet. Res.*, 1949, v10, 295.

10. Zavadovskii, M. M., Eskin, I. A., and Ovsjannikov, G. F., *Trud. Dinam. Razvit.*, 1935, v9, 75, Abstract in *Anim. Breed. Absts.*, 1936, v4, 37.

11. McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1940, v135, 473.

12. ———, *J. Biol. Chem.*, 1943, v151, 259.

13. Murphree, R. L., Black, G. W., Otto, G., and Casida, L. E., *Endocrinology*, 1951, v49, 474.

14. Beshlebnov, A. V., *Probl. Zivotn.*, 1938, v2, 73; Abstract in *Anim. Breed. Absts.*, 1938, v6, 291.

15. VanDemark, N. L., and Moeller, A. N., *Am. J. Physiol.*, 1951, v165, 674.

16. Tanabe, T. Y., Warnick, A. C., Casida, L. E., and Grummer, R. H., *J. Anim. Sci.*, 1949, v8, 550.

17. Williams, W. L., *The Diseases of the Genital Organs of Domestic Animals*. W. L. Williams, 1943, Ithaca, N. Y.

18. Cole, H. H., *Am. J. Anat.*, 1936, v59, 299.

19. Willett, E. L., Black, W. G., Casida, L. E., Stone, W. H., and Buckner, P. J., *Science*, 1951, v113, 247.

Received January 14, 1952. P.S.E.B.M., 1952, v79.

Influence of Local X-ray Treatment on Development of Jaws in Young Axolotl (*Siredon mexicanum*)* (19393)

V. V. Brunst, E. A. Sheremetieva-Brunst, and F. H. J. Figge.

From the Department of Anatomy, University of Maryland School of Medicine, Baltimore, Md.

The number and age of the experimental animals (Fig. 1) and the dosages of x-radiation are indicated in Table I.

Methods. During irradiation of the lower jaw, the upper jaw and skull were protected by a small lead plate (about 1 mm thick) which was inserted into the mouth of the anesthetized animal. The bodies of the experimental animals were protected by a lead plate 4 mm thick. Irradiations were made from the ventral aspect. During irradiation of the upper jaw, the lower jaw was similarly protected, and irradiations were made from the dorsal aspect. The conditions of irradiation were as follows: 55 kv., 3 ma. max., no filter. Some of the animals of the first group and all animals of the second group were fixed at the end of experiments. But the other animals of the first group and all of the third group were fixed at various times following irradiation. In the first group, the animals were fixed at 10, 24, 34, 43, 52, 71, and 92 days after treatment. In the third group, animals were fixed at 7, 13, 23, 33, and 54 days after treatment. The heads of animals were fixed in a saturated solution of bichloride of mercury with 5% acetic acid; embedded in paraffin; sectioned at 10 μ and stained with Böhmer's hematoxylin and eosin.

Results. In the first group, a slight retardation of growth of the treated jaw was observed 71 days after irradiation. In group III, a suppression of growth of treated jaws was observed 33 days after treatment. The difference in sensitivity to x-ray irradiation of animals of various ages was more evident long after irradiation. For example, a comparison of Group I animals 312 days after treatment with 3000 r and Group II animals 176 days after irradiation with 2000 r showed that in the latter younger group, in spite of the smaller dosage, the jaws showed a much greater suppression of growth than did the Group I animals. Moreover, the suppression of growth in the latter group became evident much sooner. In some cases, the suppression of growth of the treated jaw was so great that the animal was unable to eat and had to be fed by hand (Fig. 3 and 4). In a few animals, the upper jaws were irradiated. In these experiments, similar results were obtained, but the suppression of growth here was not as great as in the irradiated lower jaws (Fig. 2).

In accordance with our previous observations, the appearance of treated tissues in jaws fixed long after irradiation was normal (Brunst(1-3), and Brunst, Barnett, and Figge(4)). Examination of jaws of animals fixed on the day of treatment shows that they had small teeth even at this time, but these teeth disappeared completely in the treated jaws while they developed normally in the control (shielded) jaws (Fig. 6 and 7). The irradiated jaws lack teeth, but are otherwise miniature copies of normal jaws having all of their component parts.

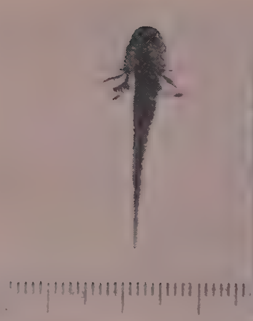
In the animals of the third group, the tissues appeared normal 7 days after treatment. In spite of this, the growth of teeth in the treated jaw was arrested. Thirteen days after treatment, some parts of the irradiated epithelium

TABLE I. Arrangement of Animals According to Age and Dosage Groups.

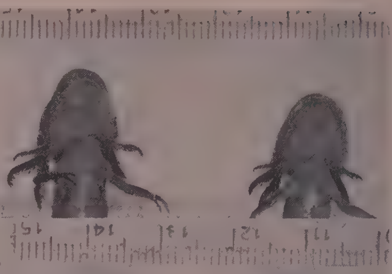
Group	No. of animals	Age in days after hatching	Size in cm (approx.)	Dosage in r
I	22	60	5	3000
II	5	20	3	2000
III	9	22	3-3½	4000

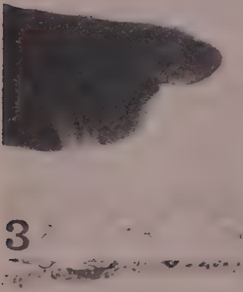
---

* This work was supported by grants from the National Cancer Institute, Public Health Service, and the Anna Fuller Fund.

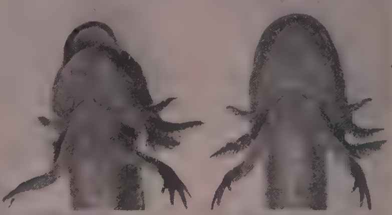
1


2

5


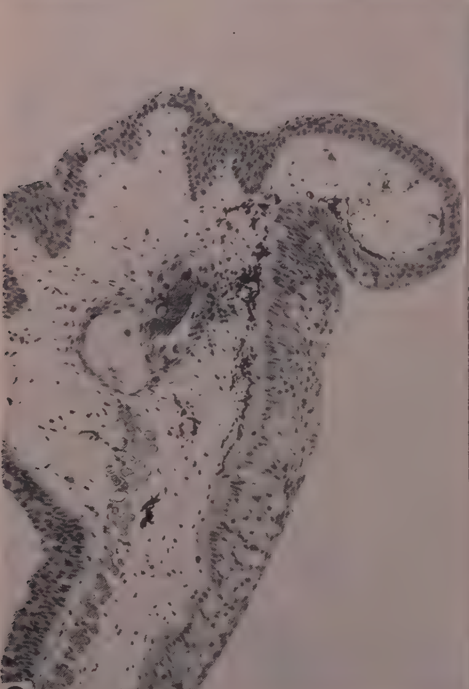
3


4

6


7

8

9


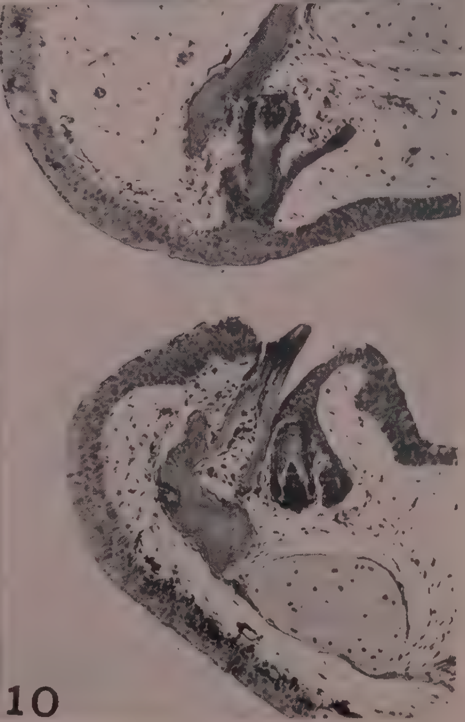
10


FIG. I. Animal on day of irradiation 20 days after hatching (second group).

FIG. 2. 250 days after irradiation of upper jaw (lateral view) with 2000 r (irradiation 20 days after hatching, second group).

FIG. 3. 176 days after irradiation of lower jaw with 2000 r (lateral view, second group, irradiation 20 days after hatching).

FIG. 4. Same animal as in Fig. 3 (ventral view).

FIG. 5. Left: Third group (irradiation 22 days after hatching) 34 days after irradiation of lower jaw with 4000 r. Right: Untreated (control) animal from same spawning.

FIG. 6. Left: Third group (irradiation 22 days after hatching) 58 days after irradiation of lower jaw with 4000 r. Right: Untreated (control) animal from same spawning.

FIG. 7 and 8. First group (irradiation 2 months after hatching) 53 days after irradiation of lower jaw with 3000 r.

FIG. 9. A sagittal section through treated jaw 120 days after irradiation with 2000 r (second group, irradiation 90 days after hatching) (× 60).

FIG. 10. A sagittal section through both jaws of a control animal of same age as in Fig. 9 (× 60).

were not normal in appearance. The mitotic activity in the treated tissues had been completely suppressed and, therefore, the formation of new enamel organs did not occur. The tissue-reaction of the irradiated jaw was especially clear 23 days after treatment. In the treated jaw at this time, there was observed a degenerating giant cell epithelium: a feature which is very typical of the primary acute reaction to irradiation (Brunst(1-3), and Brunst, Barnett, and Figge(4)). By this time, the enamel epithelium had disappeared and the resorption of teeth was in progress. There is some evidence that macrophages take an active part in the destruction of the teeth.

Animals of Group III, fixed 34 days after irradiation, still had small remnants of the teeth in some places of the treated jaws (Fig. 5). It is important to note that at this time, when the resorption of teeth is almost complete, only a small retardation of growth of the irradiated jaw is observed. The degenerating giant cell epithelium has disappeared and is now replaced with epithelium of normal appearance. In spite of great mitotic activity, this epithelium is unable to produce new enamel organs. Fig. 6 shows treated and control animals 58 days after irradiation. The retardation of growth of treated jaws is clearly evident and is much more noticeable than in the first group (Fig. 7 and 8). All remnants of teeth in the treated jaws have completely disappeared; accordingly, the histological picture of such a jaw (Fig. 9) contrasts greatly with the normal histological picture of jaws of the control animals (Fig. 10). The formation of new teeth was never observed up to approximately one year after x-ray irradiation.

Summary. Lower or upper jaws of young axolotls (age 20 to 60 days) were locally irradiated with 2000-4000 r. Suppression of growth of treated jaws was observed in all cases. The irradiated jaw is a miniature copy of the normal jaw. Tissue reaction of irradiated jaw was evident 23 days after treatment with 4000 r (primary acute reaction). The treated jaw at this time showed a typical degenerating giant cell epithelium. The enamel epithelium disappeared and resorption of teeth was in progress. Thirty-four days after irradiation there remained small remnants of teeth in parts of treated jaws. At this time, when the resorption of teeth is almost completed, only a small retardation of growth of the irradiated jaw was observed. The degenerating giant cell epithelium has disappeared and been replaced with normal-appearing epithelium, but this epithelium was unable to produce new enamel organs. Fifty-eight days after treatment the retardation of growth of the treated jaws was clearly evident, and all remnants of teeth in them completely disappeared. In the control (shielded) jaws of the same animals, the development of teeth proceeds normally. The mechanisms of tooth formation and tooth maintenance are permanently destroyed by x-ray irradiation.

---

1. Brunst, V. V., *Quart. Rev. Biol.,* 1950, v25, 1.
2. ———, *J. Morphol.,* 1950, v86, 115.
3. ———, *J. Exp. Zool.,* 1950, v114, 1.
4. Brunst, V. V., Barnett, D. J., and Figge, Frank H. J., *Am. J. Roentg. and Rad. Ther.,* 1951, v66, 420.

Received January 22, 1952. P.S.E.B.M., 1952, v79.

Virus (Herpes Simplex, Vaccinia) Studies in Embryonated Eggs with Radioactive Phosphorus.* (19394)

HARVEY BLANK,† BEN KANEDA, AND OSCAR LIU. (Introduced by Werner Henle.)

From the Department of Dermatology, University of Pennsylvania, and the Children's Hospital of Philadelphia.

A direct method for detecting the presence of virus within multicellular animals would offer many advantages over the several indirect methods now in use. The incorporation of radioactive elements within the virus offer one method of immediately detecting virus as it is disseminated in the organism and as it is absorbed onto and enters into cells. In addition radioactive "tracers" facilitate the study of the chemical changes which occur with infection. Although radioactive isotope methods have been used profitably with plant (1) and bacterial viruses(2), the study of animal viruses with "tracers" has presented difficulties. This paper and a succeeding one report on the technical problems involved and the methods used in this laboratory to prepare animal viruses with incorporated radioactive phosphorus (P^{32}). In addition other methods of using P^{32} to study virus infections in embryonated eggs are described. Taylor and Saenz demonstrated that the concentration of P^{32} inoculated into the allantoic sac of influenza infected eggs was reduced to half that found in non-infected allantoic fluids(3). Ward reported an abnormal phosphorus metabolism as demonstrated with P^{32} in embryonated eggs infected with influenza virus and with an agent isolated from the common cold(4). Rafelson, Winzler, and Pearson, using P^{32} and C^{14} glucose, found changes in the metabolism of mouse brain infected with Theiler's GD VII virus(5). Graham and McClelland reported the incorporation of P^{32} in influenza virus in chick embryos and performed chemical analyses on purified virus to demonstrate that the P^{32} was in both the phospholipid and nucleic acid fractions of the virus. Although their careful study is the first definitive one of its type, the low specific activity of P^{32} in their virus made analytical work difficult(6).

Investigation of materials and methods. Adaptation and evaluation of existing technics for handling and determining radioactive phosphorus in biological material was essential to the study. Although some of this information is known to biologists working with radioactive isotopes, it is not readily accessible, and we have been advised to report in considerable detail our basic studies which may be of help in a variety of biological problems.

Radioactivity determinations. Radioactive phosphorus was obtained from the Isotope Division, A.E.C., Oak Ridge, and one batch from the Canadian A.E.C., Chalk River. It was calibrated against a simulated P^{32} reference source (Tracerlab No. 129) and found to agree with the values supplied with each shipment. Most of the determinations of radioactivity were performed by evaporating 0.1 ml of the solution on a copper planchet and counting the activity with a mica end window Geiger Müller tube and scaler‡ It was desirable in some cases, however, to count small amounts of radioactivity in large volumes of fluid such as allantoic fluid or tissues digested with fuming nitric acid. Experiments were performed to evaluate various methods of measuring radioactivity in these biological materials in order to increase the flexibility of radioactivity determinations in tracer experiments with small amounts of P^{32}. For this study 3 ways of using Geiger-Müller tubes were compared (Fig. 1). All measurements were made with the same scaler (El-Tronics Decimal Scaler) and aliquots were prepared from the same solution of P^{32}. Wet counting of solutions was performed with a glass en-

---

* Aided by a grant from the U. S. Public Health Service.

† Present address: E. R. Squibb & Sons, 745 Fifth Avenue, New York City.

‡ We wish to acknowledge the technical assistance of Mr. Jacob Rhodes, physicist, for his aid in assuring the validity of the radioactivity determinations.

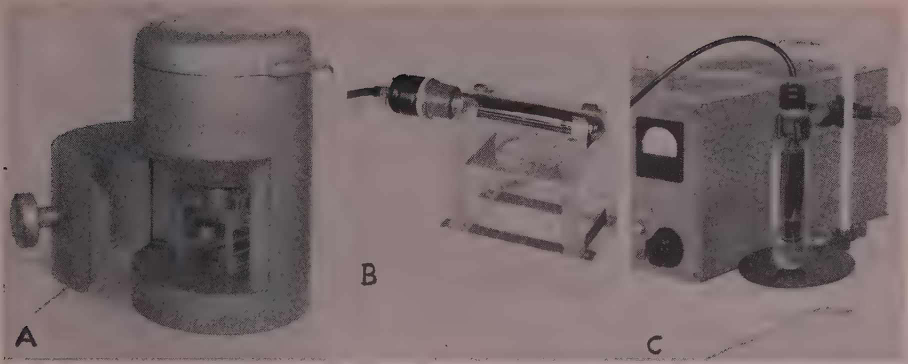


FIG. 1. Methods of measuring radioactivity with Geiger-Muller tubes. a. Mica end-window G M tube over a sample evaporated on a copper planchet. b. Glass walled G M tube over a fluid sample for "wet" counting. c. Glass walled G M tube immersed in a fluid sample for "dip" counting.

velope G.-M. tube (Tracerlab TGC-5) with a window thickness of 30 mg/cm^2 held horizontally above the dish. It was found by experiment that 5 mm of solution constituted "infinite thickness" in the dish if the distance from the surface of the fluid to the tube was kept constant. In dip counting as well, with the glass G.-M. tube immersed directly into the radioactive solution a radial thickness of 5 mm of fluid constituted "infinite thickness." Radioactive contamination of the dip tube is no problem if the tube is rinsed immediately after the determination, with water containing a few drops of phosphoric acid, then dipped in 10% nitric acid and rinsed again. A "background" count must be performed before each determination to make certain that the tube is not contaminated from the previous determination.

From the results of this comparison (Table I) it is apparent that "dip counting" will detect minimal amounts of radioactive phosphorus in dilute solution which may be impossible to measure readily with the other methods.

Decontamination of glassware. In virus or tissue culture work many cleaning agents such as sulfuric acid-dichromate solution and certain detergents are undesirable because of residual toxic films adhering to the glassware(7). Studies were conducted, therefore, with strong warm solutions of sodium metaphosphate (Calgonite^R) as a cleaning agent for virus laboratory glassware and needles. This detergent seemed suitable because it does not leave toxic films on glassware, does not damage needles, and because it contains a great excess of phosphate ions which would tend to remove adhering radioactive phosphate ions by "exchange" as well as by its detergent action. Several experiments indicate that this solution will remove P^{32} satisfactorily, either in inorganic form or when contaminated with proteins, from glassware used for solutions containing as much as 10 millicuries per ml. Glassware requires approximately 48 hours and needles approximately 96 hours of immersion in this solution to be cleaned to "background."

Sterility of P^{32} solution. Review of the technic used at Oak Ridge for the extraction of radioactive phosphorus indicates that the solution as received contains little but water, phosphate ions and very dilute hydrochloric acid(8). Because of the possibility of contamination of the material with microorganisms, the solutions as received from the U. S. Atomic Energy Commission at Oak Ridge, Tenn., and one sample prepared in the reactor at Chalk River, Ontario, Canada, were repeatedly cultured on blood agar plates, on glucose agar, and in thioglycollate media. No growth was noted in any medium after 3 weeks incubation. Although the solutions were sterile, penicillin (500 units per ml) and streptomycin (100 μg per ml) were added to obviate bacterial contamination in subsequent procedures in the laboratory.

TABLE I. Relative Sensitivity of Various Methods of Detecting P^{32} Activity in Solution. For the results below, a specific activity of 10^{-3} u C/ml was used.

Method of counting	Counter tube	Vol of solution, ml	Distance from sample surface to counter window, mm	Counts/min (corrected)	Relative sensitivity
Copper planchet with end window counter (Fig. 1a)	Type TGC-1 2.4 mg/cm² mica window	.1* evaporated	1	247	.046
5 cm glass dish with end window counter (not illustrated)	”	10†	4.3	1260	.236
5 cm glass dish with glass envelope counter horizontal above dish (Fig. 1b)	Type TGC-5 30 mg/cm² glass envelope	10‡	7.3	1171	.220
3 cm diam. cylinder, tube immersed in sol. (Fig. 1c)	”	40§	0	5340	1

* The sensitivity of the planchet method of counting can be increased by evaporation of larger volumes of solution, but the presence of solids introduces “self absorption” errors.

† This corresponds to a depth of 5 mm in a 5 cm diameter dish; greater depth does not increase the counting rate. It is not practical to get the liquid surface closer than about 4 mm to the tube window. An increase in diameter of the dish will not increase counting rate substantially because 5 cm already exceeds the window diameter.

‡ This corresponds to a depth of 5 mm in a 5 cm diameter dish. Greater depth will not increase counting rate. Some improvement will result from putting tube closer to liquid surface, and from using a larger diameter dish. An increase by a factor of 2 might result.

§ This volume corresponds to a radial thickness of liquid surrounding counting tube of 5 mm in a cylindrical container 11 cm long and 28 mm inside diameter. If a larger container is used, more solution will of course be required, but no increase in counting rate occurs after the liquid layer reaches 5 mm.

Safety precautions with P^{32}. The important precautions to prevent injury to laboratory workers handling P^{32} are defined in many Atomic Energy Commission publications and other texts. These of course must be followed. In addition, methods of handling certain problems in this project may be of interest to other virus workers. Eggs containing radioactivity were deposited in large, wide mouthed, screw capped bottles and sufficient concentrated formalin was added to prevent decomposition. They were dated and stored in a special “radioactivity decay closet” until they could be incinerated without danger. Candling of eggs containing 100 or more μc of P^{32} per egg was performed with lead rubber gloves with a glass shield between the operator and the candling box. Large volumes of dialysing fluid containing P^{32} were treated inexpensively with alkali and with calcium chloride to precipitate most of the P^{32} as calcium phosphate. The precipitate was allowed to sediment and the supernate decanted into the sink. The precipitate was poured into smaller containers and stored until decayed.

Toxicity of P^{32} for embryonated hens' eggs. To incorporate as much radioactivity as possible within the virus it was desirable to obtain a high concentration of P^{32} in embryonated eggs. Graham has reported that amounts in excess of 5640 microrutherfords (0.152 μc) per egg caused an appreciable mortality in his eggs(6). We found, however, that we could inoculate more than 100 μc into an embryonated egg without significantly increasing the embryo mortality. In an attempt to explain this discrepancy we compared the toxicity of P^{32} obtained from Oak Ridge, Tenn., with that prepared at Chalk River, Ontario, Canada, Graham's source of material. Table II indicates that there is no appreciable difference in the material obtained in 1951 from the two sources, and that in embryonated eggs from White Leghorn hens in the Philadelphia area as much as 1000 μc can be used per egg without killing a majority of the eggs during

TABLE II. Toxicity of P^{32} for Embryonated Eggs 96 Hours Following Inoculation.

	Amt of P^{32} in microcuries	Inoculated into allantoic sac, 11-day-old eggs			Inoculated into yolk sac, 9-day-old eggs		
		10	100	1000	10	100	1000
American P^{32} (Oak Ridge) 1951	No. surviving	10	10	7	9	9	—
	No. inoculated	10	10	10	10	10	
Canadian P^{32} (Chalk River) 1951	,,	9	9	7	10	8	3
		10	10	8	10	10	6

the time of the experiment. (In a personal communication, October 1951, Graham reported that he was able to confirm our findings.)

Distribution of P^{32} inoculated into normal embryonated eggs. An extensive study was made of the fate of P^{32} inoculated as a dilute phosphate solution into normal embryonated eggs as a basis for comparison with infected eggs. The P^{32} solution was inoculated into the yolk sac via a hole in the air sac end of the eggs which were subsequently incubated at 37°C. The allantoic and amniotic fluids were harvested separately. Bloody fluid was discarded. The radioactivity of 2 planchets, each containing 0.1 ml of fluid, was determined separately for each fluid from each egg. A minimum of 12 eggs of each age and for each time interval were used. For many points as many as 60 determinations were available from the controls of other experiments. The results which have been reproducible in many experiments are indicated in Fig. 2 and 3.

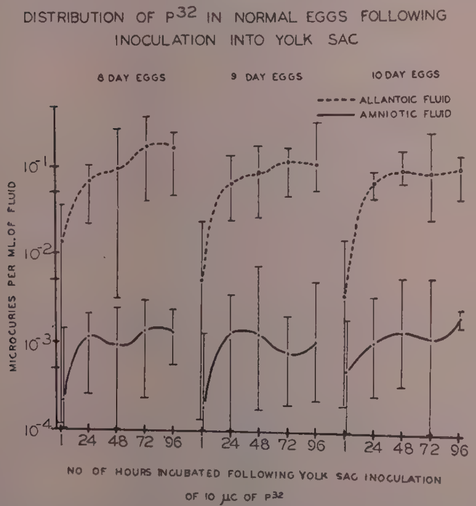


FIG. 2. Distribution of P^{32} in normal eggs following inoculation into yolk sac. A minimum of 12 eggs were used to determine each point on the curves.

Fig. 2 demonstrates that there is no significant difference within 96 hours incubation in the distribution of P^{32} injected into 8- or 9- or 10-day-old eggs. Although there is wide variation from egg to egg as indicated by the vertical lines, it is apparent that within 24 hours, there is at least a 100-fold increase in allantoic fluid P^{32} over the amniotic fluid P^{32}. Approximately 1% of the P^{32} injected into the yolk sac appears in the allantoic fluid but less than 0.01% can be found in the amniotic fluid of non-infected eggs. Additional experiments (Liu, Henle, Blank, and Kaneda, to be published), indicated that practically all of the allantoic fluid P^{32} in normal eggs can be removed by dialysis and probably is present as inorganic phosphate.

Distribution of P^{32} in virus infected embryonated eggs. The presence of radioactivity in infected tissues was determined by means of sensitive silver halide emulsions (autoradiography) as well as by counting with a Geiger-Müller tube.

Autoradiography of the chorioallantoic membrane. Autoradiograms were prepared of whole membranes and of histologic sections of membranes. The gross autograms were made by applying with pressure "No-Screen" x-ray film to the membranes which had been spread and dried on a glass lantern slide. Fig. 4 demonstrates radioactivity throughout the membrane with a concentration along blood vessels. Control exposures with non-radioactive membranes showed no "chem-

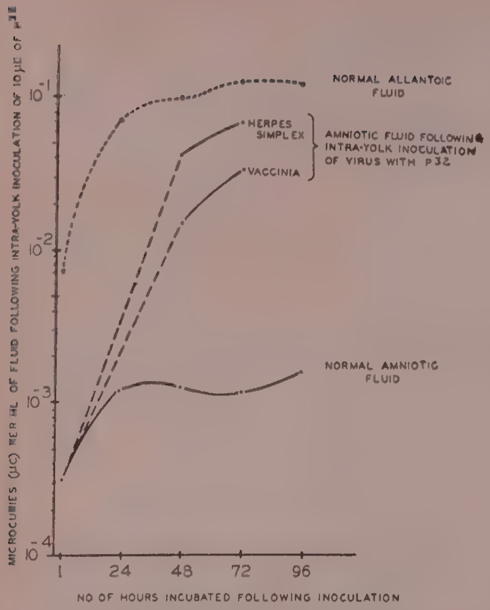


FIG. 3. Distribution of P^{32} in the allantoic and amniotic fluids of virus infected eggs as compared with non-infected eggs.

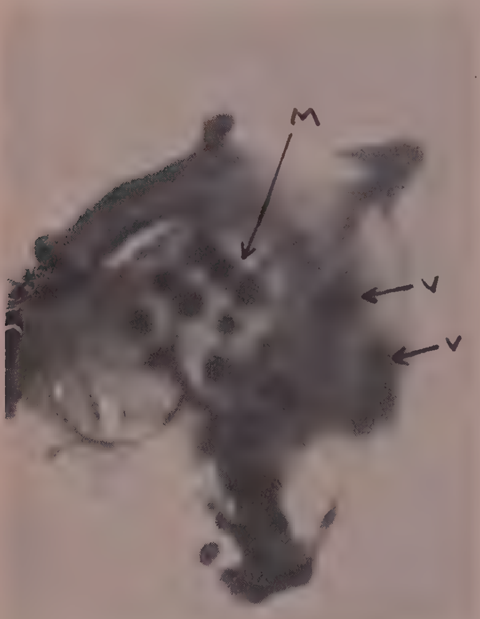


FIG. 4. Gross autoradiogram of a chorioallantoic membrane indicating areas of localization of P^{32}. In addition to the blood vessels, the P^{32} is concentrated in the "plaques." V were induced by infection with vaccinia virus and M were induced by mechanical trauma.

ical fogging" with this emulsion(9). Plaques were induced in membranes by infection with dilute vaccinia virus suspensions. Control plaques were induced mechanically by drilling small holes through the shell in a special fashion directly over the membrane. P^{32} was inoculated into the yolk sac just before infection or mechanical trauma and the eggs were incubated for 72 hours. It can be seen from Fig. 4, in which both artificial and virus induced plaques have been produced on the same membrane that a high concentration of radioactivity appears equally in both types of lesions. This probably is a non-specific phenomenon indicating merely that the rapidly growing cells which form the plaques incorporate large amounts of available phosphorus. In addition, autoradiograms were prepared of histologic sections of radioactive membranes with the method developed by the senior author which precludes the loss of P^{32} during histologic procedures(10), and these, too, show a non-specific but high concentration of P^{32} in the plaques (Fig. 5).

Partition of P^{32} in egg fluids with infection. Fig. 3 is the compilation of many experiments in which either vaccinia virus or herpes simplex virus was inoculated simultaneously with the P^{32} into the yolk sac. The vaccinia virus was a commercial calf lymph strain egg adapted in our laboratory to produce titers of 10^6 in amniotic fluid following yolk sac inoculation. The herpes simplex strain (W) was isolated from a patient, identified serologically and histologically, and egg adapted to produce titers of 10^8 in amniotic fluid following yolk sac inoculation(11). It is apparent that 48 hours after infection, at which time virus is regularly present in the amniotic fluid, there

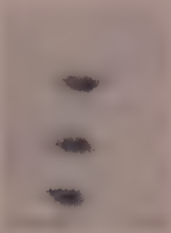


FIG. 5. Gross autoradiogram of histologic sections of chorioallantoic membrane with vaccinia induced plaque demonstrating concentration of P^{32} in the area of the plaque.

is a striking increase in the amount of P^{32} in the amniotic fluid. In practice this unmistakable change is observed as the difference, for example, between 150 counts/minute for 0.1 ml of normal fluid and 2000 counts/minute for 0.1 ml of infected fluid. In Fig. 3 the counts obtained experimentally have been converted into absolute amounts of P^{32} so that they may be compared with data obtained with other counting methods in other laboratories.

Conclusions. 1. The following observations were made on basic methods of using P^{32} in biological studies: a. P^{32} as received from Oak Ridge is usually sterile but penicillin and streptomycin can be added before direct inoculation into eggs to obviate autoclaving procedures potentially dangerous to laboratory personnel. b. Glassware and needles used for virus work can be decontaminated of P^{32} in 2-4 days with hot sodium metaphosphate (Calgonite^{R}) solutions. c. Simple methods of handling and storing millicurie amounts of P^{32} in biological materials are described. 2. Radioactive phosphorus is of relatively low toxicity for chick embryos; 500-1000 μc per egg is not lethal in 72 hours. 3. P^{32} injected into the yolk sac appears in a 100-fold greater concentration in the allantoic fluid than in the amniotic fluid in 24-96 hours. 4. The partition of P^{32} in the egg fluids is the same for 8- or 9- or 10-day-old embryos with periods of incubation of 24-96 hours following inoculation. 5. Following infection with herpes simplex or vaccinia virus there is a 10-20-fold increase in the amount of P^{32} appearing in the amniotic fluid. 6. Chorioallantoic membrane plaques, whether virus or mechanically induced, concentrate P^{32} in their cells.

1. Libby, R. L., and Madison, C. R., *J. Immunol.*, 1947, v55, 15.
2. Cohen, S. S., *Bact. Rev.*, 1951, v15, 131.
3. Taylor, R. M., and Saenz, A. C., *J. Immunol.*, 1949, v63, 319, 331.
4. Ward, T. G., *Am. J. Hyg.*, 1950, v52, 107.
5. Rafelson, M. E., Winzler, R. J., and Pearson, H. E., *J. Biol. Chem.*, 1949, v181, 583, 595; *Science*, 1950, v112, 231.
6. Graham, A. F., and McClelland, L., *Canad. J. Res. E.*, 1950, v28, 121; Graham, A. F., *loc. cit.*, 1950, 186, 271.
7. Toennies, G., *et al.*, *J. Lab. Clin. Med.*, 1951, v38, 163.
8. Lough, S. A., Isotope Division, U. S. Atomic Energy Commission, personal communication.
9. Boyd, G. A., and Board F. A., *Science*, 1949, v110, 586.
10. Blank, H., McCarthy, P. L., and DeLamater, E. D., *Stain Tech.*, 1951, v26, 193.
11. Nagler, F. P. O., *Australian J. Exp. Biol. Med. Sci.*, 1946, v24, 103.

Received January 29, 1952. P.S.E.B.M., 1952, v79,

Effects of Certain Amino Acids and Related Compounds on Propagation of Mouse Encephalomyelitis Virus.* (19395)

Harold E. Pearson, Dorothy L. Lagerborg, and Richard J. Winzler.

From the Medical Microbiological and Biochemical Departments, University of Southern California School of Medicine and the Los Angeles County Hospital, Los Angeles.

Various factors which affect the propagation of Theiler's GDVII strain of murine encephalomyelitis virus in tissue cultures have been reported(1). The experiments described below were concerned with the effects of certain amino acids, amino acid analogues and other agents on this system; preliminary data were presented elsewhere(2).

Materials and methods. The methods used have been described in detail(1). The virus used was obtained from pooled tissue culture material after 50 to 60 tissue culture passages. It was added to cultures in final dilution of 1:1,000. Cultures were made in 50 ml Erlenmeyer flasks closed with rubber stoppers and containing 3 ml of Simms' X7 solution and

---

* Aided by a Research Grant from the National Cancer Institute of the National Institutes of Health.

50-100 mg of minced mouse brain from 1-day-old mice. The initial reaction of the medium was adjusted to pH 8-9. Cultures were incubated for 2 days at 35°-36°C. The supernatant fluid obtained after centrifugation of the pooled material from 3 flasks was tested for virus content by determination of the hemagglutination titer for human red blood cells. Serial twofold dilutions were used and the end-point was read from the sediment. The titer of tissue culture supernatant fluid as tested by this method usually was 1280-2560. Virus content was also assayed by intracerebral inoculation of 7 mice each with 0.03 ml of serial 2-fold dilutions. The end-point was the highest dilution fatal for 4 or more mice during the period of observation for 21 days. Control cultures without the compound tested were run routinely. All experiments were repeated one or more times.

Results.† Of the naturally occurring amino acids only lysine, serine and ornithine inhibited virus growth at 1 mg/ml (Table I). Alanine, arginine, aspartic acid, cystine, glycine, histidine, norleucine, phenylalanine, threonine, and tryptophane were inhibitory at 3 mg/ml while leucine, isoleucine, methionine, proline and valine had no inhibitory effect at this level. Tyrosine did not inhibit at 1 mg/ml concentration but could not be tested in greater amount because of insolubility. Synthetic amino acid analogues were highly inhibitory at 1 mg/ml. Thienylalanine, furylalanine, and naphthylalanine reduced the RBC titer by 50% even at a concentration of 0.1 mg/ml. Complete inhibition with this amount was obtained with the ortho-, meta-, or para-fluorophenylalanines. The lowest concentration of lysine found to be inhibitory was 0.5 mg/ml.

Attempts were made to reverse the inhibitory effect of some of the amino acids by addition of one or more amino acids not by themselves inhibitory or by the addition of 1 mg/ml of a combination of each of the amino acids listed in Table I as noninhibitory. The latter combination, however, was inhibitory, so was not tested further. Attempted reversals of lysine inhibition by addition of individual amino acids revealed that only methionine, leucine or tyrosine partially prevented the inhibitory action.

TABLE I. Percentage Reduction in Hemagglutination and Mouse Fatality Titers of Virus in the Presence of Certain Compounds, in Comparison With Control Cultures.

Compound	% reduction in titer	
	Mouse	Hemagglut.
1 mg/ml		
L-Lysine monohydrochloride	—	50–87
DL- ” ”	—	87
L-α-Aminoadipic acid	75–94	100
D- ” ”	0	0
α-Ketoadipic acid	75	100
DL-Ornithine monohydrochloride	50–75	—
DL-Serine	50–75	87
DL-β-2 thienylalanine	87–97	100
DL-β-2 furylalanine	97	100
DL-β-1 naphthylalanine	—	80
DL-Fluorophenylalanine†	99	100
DL-Canavanine sulfate	—	100
DL-Allylglycine	0–50	0–50
DL-Methallylglycine	50–87	—
3 mg/ml*		
DL-Alanine	0–50	50–75
L-Arginine monohydrochloride	50–75	100
DL-Aspartic acid	50–75	75–94
L-Cystine	75	—
L-Glutamic acid	50	75
α-Ketoglutaric acid	0	—
Glycine	50–87	87–100
L-Histidine monohydrochloride	50–87	100
DL-Isoleucine	0	0
L-Leucine	0	0
DL-Norleucine	50–75	50–87
DL-Methionine	0	0
DL-Phenylalanine	50–75	50–87
L-Proline	0	0
DL-Threonine	50–75	100
L-Tryptophane	50–87	100
DL- ”	0	—
DL-Valine	0	0

* Noninhibitory at 1 mg/ml.

† Ortho-, meta- and para- forms equally active.

DL-β-2-thienylalanine was tested for protective activity against virus infection in mice. Ten mice weighing 10 to 12 g each received intracerebrally 0.03 ml containing 2 M.L.D. of Theiler's GDVII virus. Twenty-four hours later each was given 25 mg of thienylalanine intra-abdominally; this injection was repeated daily for 4 more days. Ten control mice were given saline instead of the drug. Nine test mice and 8 untreated controls died in 7-14 days; 1 mouse in the former and 2 in the latter group survived. Preliminary tests showed that the compound was not toxic in the amounts used since animals survived 6 successive daily doses of 25 mg without incident.

---

† We are indebted to Drs. H. Borsook and A. Haagen-Smit for supplies of α-amino and ketoadipic acids, to Dr. V. H. Horowitz for canavanine, to Dr. Karl Dittmer for the allylglycine, methallylglycine and the phenylalanine analogues, and to Dr. Carl Niemann for the ortho-, meta-, and parafluorophenylalanines.

Discussion. The reasons for the virus inhibiting action(4-8) of amino acids and amino acid analogues and for differences among them are no more apparent than they are for bacteria and fungi. In the relatively high concentrations used in the present studies only a few naturally occurring amino acids were noninhibitory. Of the naturally occurring amino acids lysine was the most inhibitory. This is particularly interesting since it has been shown that Theiler's virus has a unique effect on protein-bound lysine and histidine, in mouse brain, *in vitro*(3). The amounts of these amino acids and their incorporation of radiocarbon from glucose are markedly reduced, *in vitro,* by virus propagation while other amino acids are not affected by virus growth in this way. Possibly lysine and histidine metabolism are important in the host-virus relationship in this system. Studies of this problem are in progress.

The observation that L-α-aminoadipic and α-ketoadipic acid are as highly inhibitory as lysine is in accord with the data of Borsook, *et al.*(9), and Windsor(10) who showed that these compounds are metabolites of lysine. The inactivity of D-α-aminoadipic acid suggests that the inhibition is due to an interference with specific metabolic processes. There is no explanation at present for the partial reversal of lysine inhibition brought about by methionine and by leucine or tyrosine or for the partial reversal by methionine of the lysine inhibition of $P^{32}O_4$ incorporation into the phospholipid and protein-bound fractions of mouse brain(11). Such reversals indicate a possible metabolic relationship among these amino acids.

Summary. 1. Various amino acids and analogues in concentrations of 1-3 mg/ml inhibit the propagation of Theiler's GDVII strain of mouse encephalomyelitis virus in tissue cultures of one-day-old mouse brain. The most active amino acid was L-lysine which was inhibitory at 0.5 mg/ml. The most effective analogues tested were the ortho-, meta-, or para-fluorophenylalanines, active at 0.1 mg/ml. 2. Partial reversal of lysine inhibition of virus growth was obtained with methionine, leucine, or tyrosine.

---

1. Pearson, H. E., *J. Immunol.,* 1950, v64, 447.

2. Pearson, H. E., and Winzler, R. J., *Fed. Proc.,* 1949, v8, 409; 1950, v9, 389.

3. Rafelson, M. E., Jr., Winzler, R. J., and Pearson, H. E., *J. Biol. Chem.,* 1951, v193, 205.

4. Spizizen, J., *J. Infect. Dis.,* 1943, v73, 212.

5. Cohen, S. S., and Anderson, T. F., *J. Exp. Med.,* 1946, v84, 525.

6. Spizizen, J., Kenney, J. C., and Hampil, B., *J. Bact.,* 1951, v62, 323.

7. Brown, G. C., and Ackermann, W. W., Proc. Soc. Exp. Biol. and Med., 1951, v77, 367.

8. Ackermann, W. W., *J. Exp. Med.,* 1951, v93, 337.

9. Borsook, H., *et al., J. Biol. Chem.,* 1948, v176, 1383, 1395.

10. Windsor, E., *J. Biol. Chem.,* 1951, v192, 607.

11. Rafelson, M. E., Jr., Pearson, H. E., and Winzler, R. J., *Arch. Biochem.,* 1950, v29, 69.

Received January 30, 1952. P.S.E.B.M., 1952, v79.

Carbohydrate Content of the Human Aortic Albuminoid. (19396)

Howard Miller, Harold Haft, and Dorothy M. Kraemer.
(Introduced by J. B. Hamilton.)
From the Department of Anatomy, State University Medical Center, College of Medicine, Brooklyn, N. Y.

The albuminoid (collagen, elastin, and reticulin) of human arteries of the elastic type appears to undergo qualitative as well as quantitative aging changes. Thus, a gradual increase in collagenous substance(1), accompanied by minor changes in elastin content

(2,3) has been demonstrated with progressive age. Increases in the calcium and aspartic acid content of arterial elastin with aging have also been shown(2-6).

The demonstration that collagen from animal skin contains about 0.47% carbohydrate, probably in the form of glucose and galactose (7-9), stimulated us to investigate this carbohydrate fraction in the human aorta. Preliminary studies revealed the presence of a similar carbohydrate fraction in the albuminoid portion of the human aorta. The purpose of this investigation was therefore to determine whether or not this albuminoid carbohydrate varies with age, sex, or various pathological processes.

Materials and methods. The albuminoids were extracted by the method of Highberger (10), which was modified for our purposes. Tryptic digestion was eliminated because of the probable degradation of collagen following such treatment(11,12). It is therefore not possible to determine with certainty which albuminoid fraction contained the carbohydrate measured by our procedure, although theoretically the elastin should not be altered by this method. All samples were obtained from autopsy specimens and were refrigerated for no more than 60 hours before treatment. Only aortic tissue lying proximal to the origin of the coeliac artery was used in these experiments. The aortae were placed in absolute alcohol, shaken, and refrigerated for at least 6 hours. The alcohol was then changed and the procedure repeated for a minimum of 6 hours. Following dehydration, the samples were cut into approximately one-half inch squares, and random samples weighing about 15 g were placed in small gauze bags. From 4 to 8 of the sample bags were extracted with 750 ml of acetone in a large Soxhlet-type extractor for at least 16 hours. Following this the samples were washed with enough absolute alcohol to insure the removal of the remaining acetone, and the washings were discarded. Further extraction was then carried out for from 10 to 12 hours with 750 ml of absolute alcohol. The samples were then washed with distilled water and drained thoroughly. Treatment with 4 changes of half-saturated calcium hydroxide solution followed. This was accomplished by the use of a mechanical shaking machine. At the end of each shaking period (55 minutes) the samples were squeezed so as to express the excess calcium hydroxide before being placed in a fresh solution. The filtrate from the fourth treatment gave no precipitate on acidification with acetic acid, indicating the absence of mucoids. The samples were then washed thoroughly with distilled water, drained thoroughly, and washed with cold 0.007 N acetic acid. Four to 6 acetic acid washings were used, all washings being stirred well and thoroughly drained. After the exhaust liquid was found to be acid to litmus, several distilled water washings were used until the wash gave a negative reaction for calcium when tested with ammonium oxalate. The material was then dehydrated with several changes of absolute alcohol, washed several times with ethyl ether, and air-dried. The samples were then removed from their bags and weighed on an analytical balance. Each sample was then quantitatively transferred to a large Pyrex test tube to which was added 25 ml of 5% sulfuric acid. The tubes were placed in a boiling water bath for one hour, cooled in cold water, and the contents filtered into 100 ml volumetric flasks. The residue and filter paper were washed thoroughly with distilled water, and the flasks diluted with distilled water to the mark. After thorough shaking, 10 ml of the clear brownish solution was pipetted into a test tube containing 10 ml of 10% trichloroacetic acid solution. This procedure was necessary in order to reduce the amount of coloration. The contents of the tubes were shaken thoroughly, allowed to stand at least 10 minutes, and filtered into a dry test tube. One ml of the relatively colorless filtrate was pipetted into a test tube containing 10 ml distilled water, and the solution was shaken thoroughly. A 5 ml aliquot of this solution was then pipetted into an Evelyn macrocolorimeter tube; the tube was placed in a cold water bath. The carbohydrate was estimated by the method of Seifter *et al.*(13) and calculated on a glucose basis.

Duplicate samples from the same aorta were

TABLE I. The Albuminoid Carbohydrate Content of Human Aortae.

	No. of samples	Mean carbohydrate content, mg glucose/g albuminoid	Stand. dev.	t	Probability
Age group (yr)					
0-19	5	10.5	3.3		
20-39	9	10	5.4	.19	> .5
40-59	6	10.5	3.2	.0	1
60-69	11	11	2.2	.36	> .5
70-87	11	11.9	12.8	.24	> .5
	42	Avg 10.9	7.1		
♂	27	10.9	8.3		
♀	14	10.5	4.3	.17	> .5
Normal	8	8.9	5.8		
Pathological*	29	11.6	7.8	.91	> .05
Calcified†	9	9.4	2.5	.24	> .5

* Specimens which showed gross plaque formation, ulceration, or sclerotic changes.
† Gross observations.

analyzed in 4 cases, and a triplicate sample was determined in one case. The average percentage deviation from each mean was approximately 12%. The possibility that different parts of the same aorta are not homogeneous in respect to carbohydrate content may partially account for this figure. A 90% recovery was obtained by the addition of 6.25 mg of glucose to a duplicate sample. A total of 42 separate specimens was used for the analyses.

Results. The 42 samples were subdivided into 5 age groups and the youngest age group used as the base line for statistical analyses which were performed by Mr. G. Mestler of this department. The youngest specimen was obtained by pooling the aortae from two stillborn infants. All other samples were obtained at random from a series of autopsies. The data revealed no significant age changes in albuminoid carbohydrate content (Table I).

Likewise, no sex (Table I) differences could be found from the experimental results. All aortae were examined grossly for pathological processes. Histological examinations of these tissues will be reported in a later paper. The specimens were subdivided, on the basis of gross findings, into a normal group in which no pathological changes were observed, and a pathological group in which plaque formation, ulcerations, or calcifications were found. In comparing these 2 groups (Table I), no significant carbohydrate differences were observed. Those samples which showed definite calcified areas were also compared with the normal group. Again, no statistically significant changes could be seen from the data (Table I).

Discussion. The results of this investigation thus revealed that the albuminoid carbohydrate content of human aortae does not significantly change with age, sex, or pathology. They did, however, clearly demonstrate the presence of a constant carbohydrate fraction in the albuminoid portion of the human aorta. The exact significance of this carbohydrate fraction cannot be presently evaluated. An attempt to isolate and determine the nature of this carbohydrate fraction is being undertaken.

Summary. 1. The presence of a constantly occurring carbohydrate fraction in the human aortic albuminoid has been demonstrated. 2. This carbohydrate fraction does not appear to be altered by age, sex, or pathological changes in the aorta. 3. The possible significance of this carbohydrate has been discussed.

---

1. Troitzka-Andreva, A. M., *Arkh. biol. Nauk.*, 1930, v30, 519.

2. Lansing, A. I., Rosenthal, T. B., and Alex, M., *J. Gerontol.*, 1950, v5, 211.

3. ———, *J. Gerontol.*, 1950, v5, 112.

4. Blumenthal, H. T., Lansing, A. I., and Gray,

S. H., *Am. J. Path.*, 1950, v26, 989.
5. Lansing, A. I., Blumenthal, H. T., and Gray, S. H., *J. Gerontol.*, 1948, v3, 87.
6. Lansing, A. I., Alex, M., and Rosenthal, T. B., *J. Gerontol.*, 1950, v5, 314.
7. Beek, J., *J. Am. Leather Chem. Assn.*, 1941, v36, 697.
8. ———, *J. Research NBS*, 1941, v27, 507.
9. Grassmann, W., and Schleich, H., *Biochem. Z.*, 1935, v277, 320.
10. Highberger, J. H., *J. Am. Leather Chem. Assn.*, 1936, v31, 93.
11. Cassel, J. M., and Kanagy, J. R., *J. Am. Leather Chem. Assn.*, 1949, v44, 425.
12.———, *J. Research NBS*, 1949, v42, 557.
13. Seifter, S., Dayton, S., Novic, B., and Muntwyler, E., *Arch. Biochem.*, 1950, v25, 191.

Received February 1, 1952. P.S.E.B.M., 1952, v79.

The Measurement of Systemic Blood Flow.* (19397)

Stanley J. Sarnoff, Erik Berglund, and Philip E. Waithe.
From the Department of Physiology, Harvard School of Public Health, Boston, Mass.

Various technics for the measurement of cardiac output or large portions thereof have been proposed by Gregg and Shipley(1), Wegria *et al.*(2,6), Seely and Gregg(3), Eckstein *et al.*(4), and Bohr and Henriques (5). The method to be described below measures systemic blood flow, *i.e.* cardiac output minus coronary flow. It is relatively simple to perform and has the virtue of being accomplished by cannulation only of intrathoracic vessels.

Recent work has shown the adaptability of the Potter turbinometer to the problems of measuring total aortic flow(7). This flowmeter records pulsating flow as accurately as steady flow, and thus the necessity for using a buffer bottle or pressure reservoir in the system has been eliminated along with the undesirable shift of blood from dog to bottle and vice versa that occurs with variations in arterial pressure. Further, the sensing element is a tube 2.5 inches x 0.5 inch and significantly decreases the amount of blood required to fill the system when compared to a system using a rotameter with a buffer bottle.

Method. Mongrel dogs of both sexes were anesthetized with morphine sulfate, 4 mg per kg intramuscularly, and chloralose, 48 mg per kg, and urethane, 480 mg per kg intravenously. Dogs in excess of 14 kg were used. Smaller dogs may be used if the cannulae are made of a size smaller than those listed in Fig. 1. After a tracheotomy cannula is inserted, positive pressure breathing is maintained with a modified Starling pump. With the dog on its right side, the left ribs 3, 4 and 5 are then partially removed. The first 3 sets of intercostal vessels are ligated, but the aorta is not dissected free in order to eliminate the ooze that might otherwise occur after heparinization. The brachycephalic artery is then cleared from its point of origin to its bifurca-

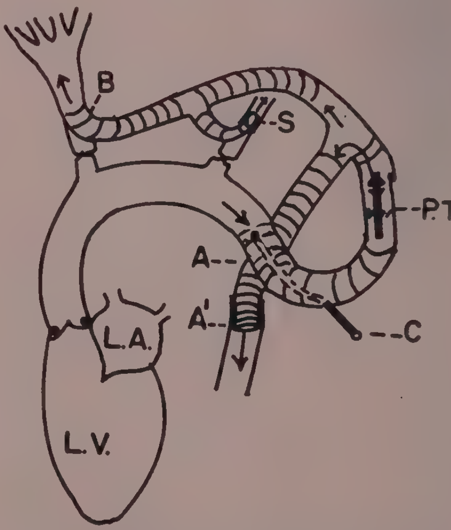


FIG. 1. Schema of aortic flow technic. See text. Desirable minimal internal diameters of cannulae are 10 mm for A, 9 mm for A′, 8 mm for B, and 3 mm for S for use in dogs from 15 to 20 kg.

---

* Aided by a grant from the National Heart Institute, U. S. Public Health Service.

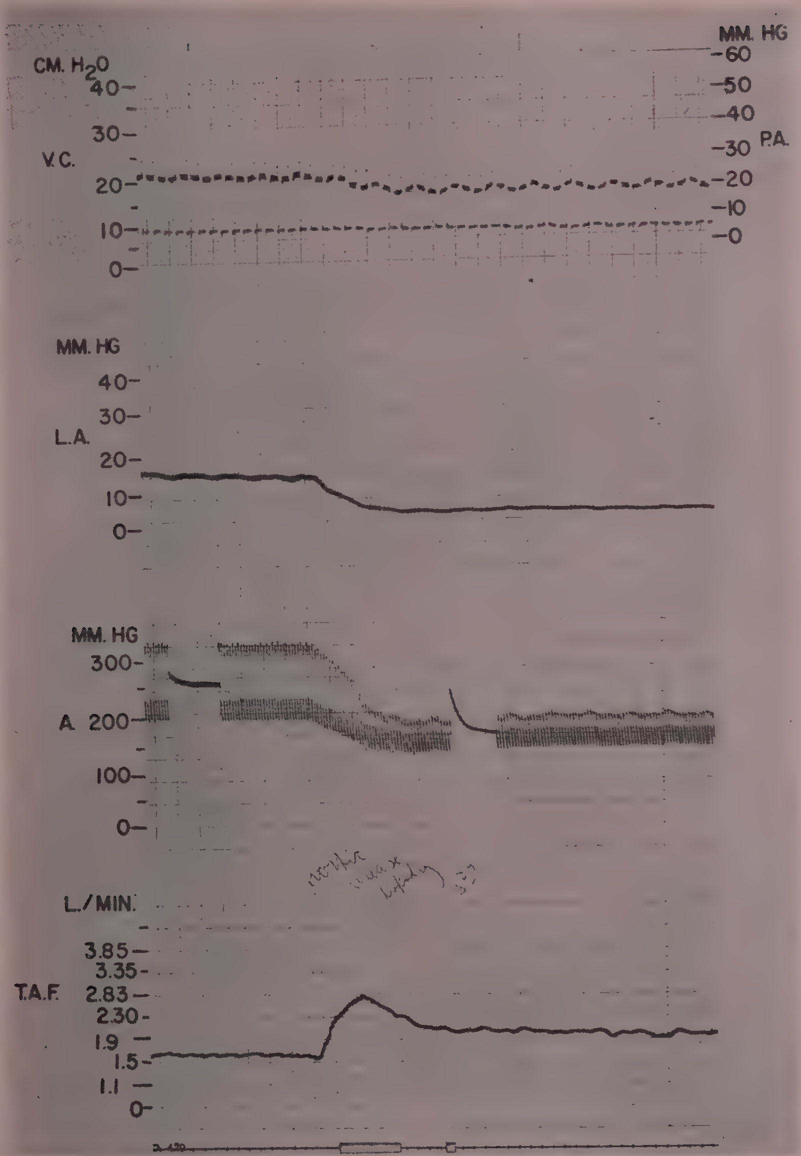


FIG. 2. T.A.F. = Total aortic flow (cardiac output minus coronary flow). A = Aortic pressure. L.A. = Left auricle pressure. V.C. = Vena cava pressure (lower tracing on top channel; scale at left). P.A. = Pulmonary artery pressure (upper tracing on top channel; scale at right). Time marker at bottom in seconds. Prior to beginning of tracing a screw clamp was placed on the tubing of outflow cannula A (Fig. 1) so as to increase aortic pressure and resistance and produce arterial hypotension distal to the screw clamp with consequent reflex vasoconstriction. Aortic clamp released at first signal. Heavy black lines = integrated (mean) pressures (V.C., P.A., L.A. and portions of aortic tracing).

tion. It is wise to ligate and divide a fine branch that leaves the brachycephalic artery posteriorly about 1 cm above its point of emergence from the aorta. The left subclavian artery is cleared for a distance of about 3 cm above its point of origin. When the preparation of the vessels is complete, 30 mg per kg of Treburon† is given intravenously. Two Blalock clamps are placed on the aorta, one just distal to the left subclavian artery and the second about 2 inches distal to the first. The proximal (A) and distal cannula (A′) are then secured in place and the system filled with blood by releasing the proximal aortic clamp and opening a side arm until no air remains (Fig. 1). The distal aortic clamp is then released, and aortic flow is reestablished. The period of occlusion of the thoracic aorta lasts between 3.0 and 4.0 minutes, and during this period the brachycephalic artery still carries blood to the brain. After about 10 minutes the brachycephalic artery is then tied off at its origin, a Blalock clamp is placed just above its bifurcation, and cannula B inserted. Flow is reestablished in between 1.5 and 3.0 minutes, during which period blood flow to the brain is maintained only by the collaterals which reach it through the still patent left subclavian artery and the right internal mammary artery that comes up from below and enters above the point of brachycephalic occlusion. Finally, either the left subclavian artery cannula (S) is inserted or the left subclavian artery is simply occluded.

The proximal aortic cannulae (A′) conducts blood to the flowmeter (PT) from which it is distributed to descending aorta, brachycephalic and sometimes also the left subclavian artery. In this way the only vessels of significant size that receive blood from the left ventricle without having it first traverse the flowmeter are the coronary arteries. The method is, therefore, one which records cardiac output minus coronary flow. To obtain registration of total cardiac output, cannulation of both coronary arteries would also be required.

Aortic pressure, proximal to the opening of Cannula A, is obtained through a length of fine vinylite tubing that pierces the wall of the tubing connected to Cannula A and extends 3 or 4 mm beyond the cannula orifice. See C in Fig. 1. The desirability of this arrangement, recently pointed out by Paine *et al.*(8), is that it avoids errors imposed by the pressure drop of the cannula orifice itself. Vena cava, pulmonary artery, and left auricle pressures are also obtained when desired by means of appropriately placed catheters connected to electromanometers(9). Fig. 2A shows the registration of total aortic flow, vena cava, pulmonary artery, left auricle, and aortic pressures during and after partial aortic occlusion.

Summary. A technic of measuring total aortic flow has been presented which essentially reestablishes cardiovascular continuity, limits vascular dead space to less than 100 cc, and is accomplished by connections with intrathoracic arteries. The use of the Potter electro-turbinometer has eliminated the pressure buffer bottle, since it follows pulsatile flow accurately.

---

† Supplied through the generosity of Dr. Elmer Sevringhaus, Hoffmann-La Roche, Nutley, N. J.

1. Gregg, D. E., and Shipley, R. E., *Am. J. Physiol.*, 1944, v142, 44.

2. Wegria, R., Guevara, R. A., and Wiggers, C. J., *Am. J. Physiol.*, 1943, v138, 212.

3. Seely, R. D., and Gregg, D. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 269.

4. Eckstein, R. W., Stroud, M., Dowling, C. V., Eckel, R., and Pritchard, W. H., *Fed. Proc.*, 1949, v8, 38.

5. Bohr, C., and Henriques, V., *Arch. de physiol. normal et path.*, 1897, v29, 590.

6. Wegria, R., Frank, C. W., Misrahy, G. A., Sioussat, R. S., McCormack, G. H., Jr., and Sommer, L. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 551.

7. Sarnoff, S. J., and Berglund, E., Manuscript in preparation.

8. Paine, R., Butcher, H. R., Smith, J. R., and Howard, F. A., *J. Lab. and Clin. Med.*, 1950, v36, 288.

9. Rappaport, M., and Sarnoff, S. J., *Fed. Proc.*, 1949, v8, 130.

Received February 4, 1952. P.S.E.B.M., 1952, v79.

Poliomyelitis Virus in Blood of Orally Infected Monkeys and Chimpanzees.* (19398)

Dorothy M. Horstmann. (Introduced by J. L. Melnick.)
From the Section of Preventive Medicine, Yale University School of Medicine, New Haven, Conn.

Although poliomyelitis virus has occasionally been isolated rather late in the experimental disease from the blood of paralyzed monkeys infected by various routes(1-3), viremia in human poliomyelitis, in spite of many attempts, has been demonstrated only once—early in the course of an abortive or non-paralytic attack(4). A possible explanation for the negative results with the human disease is that in the naturally occurring infection transient viremia occurs early, in the incubation period, or during the first phase, and by the time clinical signs of the second phase appear (paralytic or non-paralytic), it is too late to demonstrate virus in the blood. This line of reasoning prompted the present experiments, in which animals were infected by a natural route, *i.e.* orally, and blood specimens, collected during the incubation period, were tested for the presence of poliomyelitis virus.

Materials and methods. Cynomolgus monkeys (*M. irus, M. cynomolgus*) and chimpanzees (*Pan satyrus*) were used because of their known susceptibility to oral infection(5-8). Four separate experiments were carried out. In the first 3, the animals were fed the Y-SK strain (Lansing type) of poliomyelitis virus in the form of pooled monkey cords (approximately 30th passage); in the fourth, the Egypt strain, a Brunhilde type, was used in a pool of early passage monkey cords. The monkeys received 5 ml of 20% virus suspension with mashed banana on one occasion only, while the chimpanzees were fed 5 to 15 ml of the same material twice, 3 hours apart. In the first experiment, carried out in Mar. 1951, 13 monkeys were fed virus; in the second (Oct. '51), 26 monkeys were fed poliomyelitis virus, and in addition some of these received the Texas strain of Coxsackie virus and/or cortisone, 25 mg daily for 12 days. In the third experiment (Nov., '51), 4 chimpanzees were fed the Y-SK strain of virus alone, and in the fourth (Dec., '51), 15 cynomolgus were given the Egypt strain of virus. Twenty-four hours after virus feeding, 5-10 ml of blood were withdrawn from the femoral artery or vein of each animal. This was repeated daily for 5-7 days with most of the animals, although some were bled only on alternate days. All bloods were heparinized, placed in sterile lusteroid tubes within larger tubes, and spun at 2000 rpm before being frozen on dry ice. When prepared for inoculation, the middle section of the frozen blood was cut out, so that the 1 cc inoculum contained equal amounts of plasma and RBC, and the entire buffy coat, or at least the majority of the WBC from about 6 cc of blood. Tests for virus in these samples were carried out by intracerebral inoculation of rhesus monkeys, one animal for each specimen. If the test monkey failed to show signs of disease after 3-4 weeks, it was reinoculated with the same material a second time. Test animals were observed for 4 weeks after the last virus inoculation; those which developed paralysis and any which showed questionable signs were sacrificed for histological study.

Results. A summary of the findings appears in Table I. In the 1st experiment, 4 of 13 monkeys became paralyzed after feeding, the incubation periods ranging from 11 to 32 days. All of the bloods which had been collected from the paralyzed animals were tested, but only one monkey (Cy 5372) proved to have viremia: a specimen of its blood collected 5 days after virus feeding, on the 6th day before the appearance of paralysis, produced typical poliomyelitis in a rhesus monkey. In the second experiment, only 1 of the 26 animals developed paralysis; this was Cy 5965, which had been given daily injections of cortisone for 12 days following virus feeding. Blood specimens from this animal, collected 4 and 5 days after feeding, on

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Viremia in Chimpanzees and Monkeys After Ingestion of Poliomyelitis Virus.

Date	Strain fed	No. animals paralyzed	Animal	Results of tests for virus in blood—Days after virus ingestion							Day of paralysis
				1	2	3	4	5	6	7	
3/31	Y-SK	4/13*	Cy 5372	—	—	—	—	+			11
			5367	—	—	—		—			32
			5423	—	—	—		—			11
			5432	—			—	—			12
[illegible]	"	[illegible]/26	Cy [illegible]		—		+	+			11
			5795					+			No apparent disease
			5803				+	+			
			Chimpanzees								
11	"	0/4	Karin	—	—	—	+	+	—	—	No apparent disease
			Ingrid	—	—	—	—	+	—	—	
			Asta	—	—	—	+	+			
			Lisa				—	—			
[illegible]	Egypt	10/15†	Cy [illegible]				+	+	+		8
			5938	—	—		Inc.	+	+		9
			[illegible]	—	—	—	+	+	+		9

* Numerator indicates number paralyzed; denominator, number fed.

† The testing of the bloods of only 3 of the paralyzed monkeys in this group has been completed to date.

+ and — indicate positive and negative tests for virus. Inc. = incomplete test.

the 6th and 7th days before the appearance of paralysis, both yielded positive tests for poliomyelitis virus. Two other monkeys (5795, 5803), although they did not show evidence of disease, were nevertheless infected, as indicated by the development of specific neutralizing antibodies. The blood specimens of these animals, collected on the 4th and 5th days after virus ingestion, were also found to contain poliomyelitis virus. The administration of cortisone or cortisone and Coxsackie virus in this experiment did not increase the incidence of paralytic disease. Of the 4 chimpanzees fed virus in the third experiment, 3 proved to have viremia on the 4th and 5th days after virus ingestion.‡ As is usual in these animals, none developed signs of the disease. Ten of the 15 monkeys fed the Egypt strain of poliomyelitis virus in the fourth experiment developed paralysis with incubation periods ranging from 8 to 12 days. Blood specimens from several of these have been tested and viremia has been detected on the 4th, 5th, and 6th days after virus ingestion.

All blood samples collected from chimpanzees and paralyzed monkeys during the first 3 days after feeding failed to produce poliomyelitis in the test animals; 6th and 7th day bloods from two chimpanzees were also negative.

Comment. Mention has already been made that poliomyelitis virus has been isolated from the blood of experimentally infected monkeys, late in the paralytic disease. It has been suggested that that phenomenon is part of a severe or overwhelming infection, and is perhaps a terminal event. On the other hand, the demonstration of poliomyelitis virus in the blood early in the incubation period in animals experimentally infected by the oral route with both Brunhilde and Lansing types, is a new finding. It suggests the possibility that viremia may occur in the incubation period of the human disease. Since it is known that antibodies appear in the blood of cynomolgus monkeys(9) and chimpanzees(10) as early as 7 to 10 days following ingestion of virus, the first 5-7 days after virus feeding was the period upon which attention was concentrated. Whether or not viremia occurs later in the incubation period of the experimental disease has not yet been determined. In the experiment on chimpanzees (No. 3), the viremia was transient and limited to the 4th and 5th days. In the human disease, as in orally infected monkeys, antibodies are already present at the time of onset of clinical symptoms(11,12), which suggests that the time to look for viremia in man is early in the incubation period.

---

‡ In a subsequent experiment, as yet incomplete, these same chimpanzees which had developed viremia following infection with the Y-SK strain were fed Egypt strain (Brunhilde type). To date, 2 of the 4 have been found to have viremia on the 5th and 6th days after virus ingestion, indicating that viremia can occur in the same animals at least twice, with 2 different immunological types of virus.

Summary. Seven of 10 cynomolgus monkeys and 3 of 4 chimpanzees have been found to have viremia early in the incubation period following oral infection with poliomyelitis virus. Viremia was present in animals infected with a Brunhilde type (Egypt) as well as a Lansing type (Y-SK), and persisted in some animals over the 4th, 5th, and 6th days after virus ingestion. Virus was not isolated from blood specimens collected during the first 3 days after feeding, nor on the 7th day, in the two instances in which it was tested. The interval between viremia and the appearance of paralysis varied between 3 and 7 days in the cynomolgus monkeys.

---

1. Flexner, S., and Lewis, P. A., *J. Exp. Med.*, 1910, v12, 227.

2. Sabin, A. B. and Ward, R., *Science*, 1942, v95, 300.

3. Melnick, J. L., Proc. Soc. Exp. Biol. and Med., 1945, v58, 14.

4. Ward, R., Horstmann, D. M., and Melnick, J. L., *J. Clin. Invest.*, 1946, v25, 284.

5. Kling, C., Levaditi, C., and Lépine, P., *Bull. Acad. Med.*, Paris, 1929, v102, 158.

6. Burnet, F. M., Jackson, A. V., and Robertson, E. G., *Austral. J. Exp. Biol. and Med. Sci.*, 1939, v17, 375.

7. Howe, H. A., and Bodian, D., *J. Exp. Med.*, 1945, v81, 255.

8. Melnick, J. L., and Horstmann, D. M., *J. Exp. Med.*, 1947, v85, 287.

9. Von Magnus, H., *Separatum. Acta Pathologica*, 1950, v27, 222.

10. Horstmann, D. M., and Melnick, J. L., *J. Exp. Med.*, 1950, v91, 573.

11. Hammon, W. McD., and Roberts, E. C., Proc. Soc. Exp. Biol. and Med., 1948, v69, 258.

12. Steigman, Alex. J., and Sabin, A. B., *J. Exp. Med.*, 1949, v90, 349.

Received February 6, 1952. P.S.E.B.M., 1952, v79.

Measurement of Total Body Water in the Dog with Antipyrine.* (19399)

Marcia Herrold and Leo A. Sapirstein.

From the Department of Physiology, Ohio State University, Columbus, O.

Body water has been measured in man(1) and in other animals(2,3) by determining the volume of distribution of antipyrine, which appears to equilibrate with all compartments of body water. The originators of the method have, however, questioned the usefulness of this technic in the determination of body water in the dog, for although antipyrine is present in all tissues and organs in this species in amounts proportional to the water content, it is so rapidly destroyed in the dog that it was thought questionable that equilibration could be attained before nearly complete destruction of administered antipyrine.

In preparation for some experiments involving the determination of body water in human subjects, we attempted to study the technic in dogs. In agreement with previous workers(2) we found that blood antipyrine concentrations in the dog decreased at a rate some 5 times greater than the corresponding decrease in man. Since equilibrium in man is not attained before one to 2 hours, it appeared probable that destruction in the dog might be complete before adequate mixing in the body fluids could be accomplished. However, in investigating this question further, we found that the rate of equilibration of antipyrine with body fluids in the dog was so rapid that it compensated for its rapid destruction and made feasible the determination of body water in this species by the antipyrine method.

Methods. The dogs used were mongrel

---

* Supported by a grant for Cancer Research by the Ohio Department of Health.

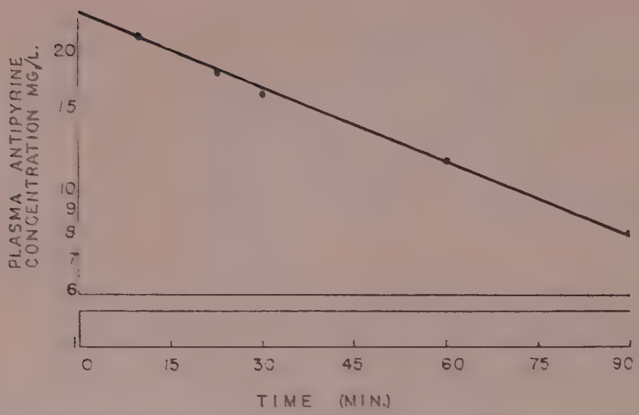


FIG. 1. Typical antipyrine concentration-time curve in the dog (plot on semilog paper).

dogs of both sexes which were chosen to represent a large range of body size and nutritional status. Some animals appeared to be suffering from severe inanition. All animals were fasted overnight and antipyrine was administered intravenously on the following morning at doses of 250 mg in 25 ml of saline. A control blood sample was taken from the external jugular vein just before the injection, and further blood samples were taken at 10, 20, 40, 60, and 90 minutes after the administration of the antipyrine. The concentrations of antipyrine were then determined on the protein free plasma by the method of Brodie *et al.*(2), using the Beckman DU spectrophotometer with the UV light source but with glass rather than quartz cuvettes. Optical densities of 0.020 to 0.035 were typical for the blanks, and the dose of antipyrine administered was sufficient to give optical densities of 0.100 to 0.150 in dogs of moderate size (10 kilos) at 60 minutes when plasma was diluted 1:4. The values of plasma antipyrine obtained were plotted on semilog paper against time, and the concentration at zero time was extrapolated from the decay curve. Anesthesia (intravenous pentobarbital sodium) was used in some dogs. The results did not appear to be significantly affected by this anesthetic.

Results. The antipyrine concentration-time curve obtained was similar in all dogs, though the rate of decay showed some variability, ranging from 20% to 60% per hour with an average of 31% per hour. A typical curve is shown in Fig. 1.

Examination of our curves shows that in all cases even the earliest plasma samples taken (10 minutes) fall on the line extrapolated from the later periods. If mixing were inadequate at this time, one would expect to find the earlier concentrations higher than the extrapolated linear curve. The fact that they are not so indicates that antipyrine equilibrates with the body fluids in the dog in the surprisingly short time of less than 10 minutes.

The rapid equilibration of antipyrine with the body fluids in the dog makes the determination of antipyrine space feasible in this animal despite the high destruction rate of this substance. We have found that samples taken at 20, 40, 60, and 90 minutes will ordinarily give a sufficient number of usable points to enable construction of a linear decay curve with assurance.

By use of this method, the antipyrine space of 8 apparently normal dogs in good nutritional status was found to range from 53.9 to 67.9% of the body weight. The average value was 59.9%. This value correlates excellently with the reported values for the water content of dogs determined as deuterium oxide space of 63%(4) and adds further support to the view that antipyrine is distributed throughout the body water. Emaciated dogs showed somewhat higher values for antipyrine space, values of 75.0, 75.6, and 79.5% being obtained in 3 such animals. This finding is consistent with Behnke's observation(5) that body water is a constant proportion of lean body mass, and that it is reduced in relation

TABLE I. Volume of Distribution and Rate of Destruction of Antipyrine in the Dog.

Sex	Wt, kilo	Antipyrine space, %	Decay rate, %/hr
♀	10.4	55.2	31
♂	8.2	60.8	28
♀	17.3	60.1	23
♀ *	13.3	75	29
♀ *	18.4	79.5	46
♀	14.8	53.9	31
♀ *	4.2	76.5	30
♀	16.9	56.8	21
♂	24	67.9	60
♂	10	59.1	24
♂	18.5	66, 66.4†	20

* Very lean or emaciated dogs.
† Repeat determination after a 3 week period.

to total body mass in obese animals and men. The results in 11 dogs are given in Table I.

Summary and conclusions. 1. Intravenously injected antipyrine is destroyed in the dog at the rate of 20 to 60% per hour. 2. Nevertheless, antipyrine space may be used for the determination of total body water in the dog, because of the fact that the distribution of antipyrine appears to be essentially complete after 10 minutes. 3. Dogs in adequate nutritional status are found to have antipyrine spaces of 53.9% to 67.9%. The average value obtained from 8 normal dogs was 59.9%. 4. Inadequate nutrition and emaciation result in an increase in the antipyrine space, a result consistent with the idea that body water is related more closely to lean body mass than to body weight.

---

1. Soberman, R., Brodie, B. B., Levy, B. B., Axelrod, J., Hollander, V., and Steele, J. M., *J. Biol. Chem.*, 1949, v179, 31.

2. Brodie, B. B., Axelrod, J., Soberman, R., and Levy, B. B., *J. Biol. Chem.*, 1949, v179, 25.

3. Kraybill, H. F., Hankins, O. G., and Bitter, H. L., *J. Applied Physiol.*, 1951, v3, 681.

4. Smith, H. W., *The Kidney*, Oxford University Press, New York, 1951.

5. Behnke, A. R., Feen, B. G., and Welham, W. C., *J.A.M.A.*, 1942, v118, 495.

Received February 6, 1952. P.S.E.B.M., 1952, v79.

Passive Immunity in Poliomyelitis. IV. Protection of Rhesus Monkeys Against Cerebral Challenge.* (19400)

A. J. RHODES, F. T. SHIMADA, EINA M. CLARK, W. WOOD, AND R. C. RITCHIE.
(Introduced by R. C. Parker.)

From the Connaught Medical Research Laboratories, University of Toronto, and The Hospital for Sick Children, Toronto.

During the last 20 years, several workers have investigated the protective properties of immune serum from human and animal sources in experimental poliomyelitis. The significance of much of this earlier work is difficult to assess, as technics for the accurate titration of virus pools and immune sera had not been developed, the existence of distinct immunologic types of virus was unsuspected, and often the number of animals used was too small for the results to have statistical value. The experiments of Kramer(1,2), however, indicated that human convalescent serum exerted a significant degree of protection in mice challenged cerebrally with the Lansing strain. Interest in this subject was revived when it was shown that gamma globulin prepared from human plasma protected mice against the Lansing strain(3). Bodian(4) demonstrated the presence in this material of antibody to all three immunologic types of poliomyelitis virus. Later he showed that some protection against an intramuscular challenge was achieved in rhesus monkeys inoculated intramuscularly with 2 ml of gamma globulin per kilogram body weight(5). We have shown that mice can be protected against a cerebral challenge of Lansing mouse virus by the inoculation of monkey immune serum, as well as plasma and placental gamma globulins(6). The present paper describes 3 passive pro-

---

* Aided by a Grant from The National Foundation for Infantile Paralysis.

tection experiments in monkeys, the virus challenge being given intracerebrally with the object of paralyzing 90-100% of controls, and thus making easier the evaluation of any sparing effect. In each experiment the virus challenge was given at a time when large amounts of circulating antibody were present. It is possible that the mechanism of protection depends on the neutralization of virus at the site of inoculation, because of the leakage of antibody from ruptured vessels. We consider, however, that this possibility does not detract from the theoretical value of the results.

Materials and methods. Monkeys. Rhesus monkeys were used in all experiments. They weighed from 5 to 11 lbs and were obtained from Okatie Farms, South Carolina. All were shown to be tuberculin negative. *Sera and gamma globulin.* Plasma gamma globulin used in the preliminary experiments was supplied by the American National Red Cross; the 50% neutralizing titer determined in mice for the Lansing strain was 3.5 logs ($10^{-3.5}$). Placental gamma globulin was generously provided by Lederle Laboratories Division, American Cyanamid Company, and contained at least 16% gamma globulin. The average 50% neutralizing titer against Lansing virus was 3.5; this material exerted a powerful protective action in mice against intracerebral challenge of Lansing virus(6). The pooled immune monkey serum was obtained from rhesus monkeys injected every 2 weeks by the intramuscular route with Lansing virus incorporated either in distilled water or adjuvants (7). The 50% neutralizing endpoint of the serum pool used in Experiment 2 was 4.5 and in Experiment 3, 3.86. No Lansing antibody was detected in the pools of normal monkey serum. *Virus pools.* Lansing virus monkey pool No. 9 was used throughout for the challenge of monkeys(7). It was prepared from the cord enlargements of monkeys harvested on the first day of paralysis following inoculation of a $10^{-2.0}$ dilution of monkey cord pool No. 8. A titration of pool No. 9 was carried out shortly after its preparation, by inoculation in 5 groups of 10 monkeys, and the PD_{50} was found to be 5.2. *Histologic examination of monkeys.* Histologic examination was carried out on all test animals, and on those control animals in which typical paralyses were not observed. The brain and cord were removed from each animal, fixed in formalin, and sections of cortex, basal ganglia, brain stem, cerebellum, and several levels of spinal cord were examined microscopically.

TABLE I. Lansing Antibody Levels in Monkeys after 20 ml (Intravenous Injection) of Plasma Gamma Globulin Approximately 4 ml/lb body wt.

		50% neutralizing titer of monkey serum at following times*			
No.	Wt	Before inj.	24 hr	48 hr	72 hr
A242	5 lb, 2 oz	0	2.65		
A243	5 15	0	2.72	1.95	
A244	5 3	0	2.79	2.72	1.9

* Sera tested with 17 LD_{50} of Lansing virus.

TABLE II. Lansing Antibody Levels in Monkeys after 20 ml (Intramuscular Injection) of Plasma Gamma Globulin Approximately 3 ml/lb body wt.

		50% neutralizing titer of monkey serum at following times*				
			Days			
No.	Wt	Before inj.	4	7	10	14
A293	7 lb, 8 oz	0	2.76			
A294	6 14	0	3.1	2.72		
A296	7 12	0	2.65	2.72	2.16	1.67

* Sera tested with 17 LD_{50} of Lansing virus.

Preliminary experiments in monkeys. Certain preliminary experiments were carried out in order to estimate the neutralizing titers likely to be obtained in monkey serum shortly after the intravenous or intramuscular injection of gamma globulin. In Table I are shown the Lansing virus antibody levels in 3 monkeys at various intervals after the intravenous injection of approximately 4 ml of plasma gamma globulin per pound of body weight.

In Table II are shown the Lansing virus antibody levels in the serum of 3 larger monkeys after the intramuscular injection of approximately 3 ml of plasma gamma globulin per pound of body weight. It will be seen that antibody titers in the region of 3.0 were obtained 4 days after the inoculation of 3 ml of gamma globulin per pound of body weight intramuscularly, whereas such a level was only found 24 hours after an injection of approximately 4 ml per pound of body weight intravenously. A serum antibody titer of 3.0 was considered by Morgan(8) to represent the

TABLE III. Passive Protection Experiment with Placental Gamma Globulin Intravenously (Experiment 1).

No. of animals and designation	Avg wt of monkeys	Prophylactic admin. intrav. day before challenge	Challenge inoculum (thalamic)	Deaths from causes other than poliomyelitis	Polio-myelitis rate
10 control	5 lb, 14 oz	0		0/10	10/10
10 test	5 14	5 ml (.8 ml/lb body wt)	160 PD_{50} (.8 ml of 10^{-3} dilution of Lansing cord pool #9)	0/10	9/10
10 ”	6	25 ml (4 ml/lb body wt)		1/10 (meningitis)	8/9

TABLE IV. Passive Protection Experiment with Immune Monkey Serum Intramuscularly (Experiment 2).

No. of animals	Avg wt of monkeys	Monkey serum admin. intramusc. day before challenge	Challenge inoculum (thalamic)	Deaths from causes other than poliomyelitis	Poliomy-elitis rate	Avg incuba-tion days
20 control	6 lb, 8 oz	5 ml normal serum per lb body wt	50 PD_{50} (.8 ml of $10^{-3.5}$ dilution of Lansing cord pool #9)	1/20 (meningitis)	18/19	7
20 test	6 6	5 ml immune serum per lb body wt		4/20 (3 meningitis, 1 diarrhea)	4/16	14

minimum level in vaccinated animals to be associated with resistance to cerebral challenge; our preliminary experiments suggested that such a level could be achieved by parenteral administration of gamma globulin or immune serum of high titer.

Protection experiments in monkeys. In these experiments monkeys were assigned to the test and control groups at random. The prophylactic agent or normal control serum was given intramuscularly. Both test and control groups were challenged thalamically with Lansing virus on the day after serum inoculation, and were observed daily for a period of 6 weeks or until the onset of paralysis. Animals were killed only when there was obviously no hope of recovery; and histologic examination was performed on all test animals, and on control animals if the diagnosis appeared doubtful. Rectal temperatures were recorded daily on the test animals, and small samples of blood were taken for antibody study, from both test and control animals, 4 days after injection of the gamma globulin or serum, and at weekly intervals for 6 weeks.

Exp. 1. Thirty monkeys were used in this experiment. Ten animals in the control group received no injection of gamma globulin, 10 animals in the test group received 5 ml, and another 10 animals 25 ml of gamma globulin intravenously on the day before thalamic challenge with 160 PD_{50} of Lansing virus. The results of this experiment are recorded in Table III, from which it will be seen that all 10 controls succumbed to poliomyelitis. Two of 19 animals injected with gamma globulin failed to develop paralysis, and as one paralyzed monkey in the test group receiving 5 ml survived for 6 weeks, 3 of 19 monkeys in the test groups escaped a fatal paralysis.

Exp. 2. In this experiment, 20 control and 20 test animals were used, the former receiving 5 ml of normal monkey serum per pound body weight, and the latter immune monkey serum (titer 4.5) in the same dosage. All monkeys were challenged the following day with 50 PD_{50} of Lansing virus. Table IV shows the results of this experiment. It will be noted that 18 out of 19 control monkeys contracted poliomyelitis, whereas only 4 out of 16 test animals did so; there was also a striking contrast in the average incubation periods of the 2 groups. Twelve animals in the test group remained healthy for 6 weeks and histologic

TABLE V. Passive Protection Experiment with Immune Monkey Serum Intramuscularly (Experiment 3).

No. of animals	Avg wt of monkeys	Monkey serum admin. intramusc. day before challenge	Challenge inoculum (thalamus)	Deaths from causes other than poliomyelitis	Poliomyelitis rate	Avg incubation days
20 control	9 lb, 11 oz	3 ml normal serum per lb body wt	100 PD_{50} (.8 ml of $10^{-3.2}$ dilution of cord pool #9)	0/20	20/20	5.9
20 test	9 15	3 ml immune serum per lb body wt		2/20 (1 trauma, 1 brain abscess)	3/18	17.3

examination revealed no poliomyelitis. The survival of 12 out of 16 monkeys points to a high degree of protection (75%) afforded by the high titer homologous immune serum. Even if it is assumed that the 4 animals dying from other causes might have acquired poliomyelitis if they had survived long enough to become infected, the survival rate is still 12 out of 20 (60%).

Exp. 3. The design of this experiment was identical with that of Experiment 2, except that an immune serum pool of slightly lower titer was employed (3.86) and 3 ml per pound of body weight were given. A slightly greater challenge (100 PD_{50}) was employed. The results are recorded in Table V, where it will be seen that all 20 control animals developed poliomyelitis while only 3 of 18 test animals contracted the infection. There is again a marked difference in the average incubation periods in the 2 groups. Fifteen out of 18 test animals survived for 6 weeks, and histologic examination revealed no evidence of poliomyelitis infection.

Neutralization tests are at present being carried out on the sera obtained in Exp. 2 and 3 in order to correlate resistance to cerebral challenge with serum antibody level. The results of these tests will be reported separately.

Summary. 1. Lansing virus serum antibody titers in the range of $10^{-2.0}$ to $10^{-3.0}$ can be achieved in monkeys shortly after the administration of gamma globulin intramuscularly or intravenously. 2. Gamma globulin injected intravenously failed to protect a significant number of monkeys against an intracerebral challenge of 160 PD_{50} of Lansing virus. 3. In 2 further experiments, the majority of monkeys receiving high titer immune monkey serum were protected against an intracerebral challenge of 50 and 100 PD_{50} of Lansing virus respectively. In one experiment only 4/16 test animals became paralyzed as compared to 18/19 controls. In the other experiment 3/18 test animals developed poliomyelitis as compared to all of 20 controls.

---

1. Kramer, S. D., PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 287.

2. ———, *J. Immunol.*, 1943, v47, 67.

3. Enders, J. F., *J. Clin. Invest.*, 1944, v23, 510.

4. Bodian, D., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 259.

5. ———, *Amer. J. Hyg.*, 1951, v54, 132.

6. Rhodes, A. J., and Clark, E. M., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 379.

7. Rhodes, A. J., Clark, E. M., and Shimada, F. T., *Can. J. Med. Sci.*, 1952, February.

8. Morgan, I. M., *J. Immunol.*, 1949, v62, 301.

Received February 6, 1952. P.S.E.B.M., 1952, v79.

Electrophoretic Studies on Serum and Plasma of Control and Vitamin Deficient Monkeys.* (19401)

Louis D. Greenberg, Urs J. P. Hoessly,† Robert Brooks, and James F. Rinehart.
From the Division of Pathology, University of California School of Medicine, San Francisco.

In connection with the study of various vitamin deficiencies in rhesus monkeys, we investigated the serum proteins of these animals, both by electrophoretic and by chemical means. It was believed that fundamental work of this nature employing highly purified diets with controlled intakes of the vitamins might be of value in interpreting abnormalities observed in the electrophoretic patterns of humans.

Electrophoretic analysis of plasma or serum of monkeys has been investigated in a few animals by Deutsch and Goodloe(1), and by Moore(2). Monkey plasma was shown by Deutsch *et al.* to contain, in addition to albumin and fibrinogen, three alpha globulin components and a component with a mobility greater than that of albumin which they have designated as "f". This component has been observed frequently in the patterns to be described in this paper.

Analyses have been carried out on one or more samples of serum or plasma from a large series of animals, including controls and monkeys subjected to various vitamin deficient regimens.

TABLE I. Composition of Basal Diets.

Constituents	Diet.* (S)	(HP)	(MF)	(AP)
Casein	18	36	18	
α protein of soy beans	—	—	—	18
Powdered sucrose	73	55	59	73
Corn oil	2	2	2	2
Hydrogenated cotton seed oil (Crisco)	—	—	6	
Salts (Hawk & Oser)	4	4	4	4
Methionine			—	.3

* (S) Standard; (HP) High protein; (MF) Moderate fat; (AP) α protein.

Supplements: A daily vitamin tablet containing: Niacin 5 mg, riboflavin 1 mg, thiamine chloride .5 mg, calcium pantothenate 3 mg, choline dihydrogen citrate 100 mg, Paba 100 mg, inositol 100 mg, ascorbic acid 25 mg, pteroyl glutamic acid 110 γ, biotin 10 γ. Pyridoxine HCl was administered daily or twice weekly in doses equivalent to 1 mg daily. In addition each animal received by mouth once a week 10 drops of vitamin A and D concentrate (100000 I.U. vit. A; 10000 I.U. vit. D) and 5 drops mixed natural tocopherols. Choline deficient and control animals on diet AP received 3.5 γ vit. B_{12} *per os* twice weekly. Choline deficient animals received no methionine.

Experimental. The rhesus monkeys were, in most instances, young animals weighing 1.5 to 3.0 kg when they were purchased. In Table I are listed the composition of the basal diets and the supplements employed. The methods of handling and feeding the animals have been described(3). Deficiency was induced by omission of the appropriate vitamin from the supplement. Blood was usually drawn in the morning before the day's food ration was introduced into the cage. When plasma was desired, Wintrobe oxalate mixture was employed as anticoagulant. The serum or plasma was separated by centrifugation and diluted with 2 to 3 volumes of sodium citrate-veronal buffer(1) (pH 8.6, u = 0.1), depending upon the protein concentration. The diluted samples were dialyzed according to the method of Reiner and Fenichel(4) for a period of 2 hours at room temperature with one change of buffer at the end of the first hour. The electrophoretic technic of Longsworth(5) was followed using a microcell of 2 ml capacity whose use has already been described by Ross *et al.*(6), and by Cohen and associates(7). Electrophoresis was carried out at 2 to 3°, generally with a constant current of 7 to 8 ma. and a field strength of 9 volts per cm, for periods varying from 3000 to 6780 sec., depending on the time required to give maximum development of the pattern. The patterns were photographed by the scanning method of Longsworth(8), and the areas of the peaks were estimated by the method of Tiselius and Kabat(9). Because of their

---

* Aided by grants from the American Cancer Society and the National Heart Institute of the U. S. Public Health Service.

† Present address, University of Utah School of Medicine, Salt Lake City.

greater sharpness, the ascending patterns were used for all calculations. Total protein, albumin and globulin were determined chemically by the biuret method as modified by Wolfson *et al.*(10) with the exception that the globulin was precipitated by 26% Na_2SO_4 (11).

Results. Data on the nutritional status, diet, duration on the regimen along with the comparative results of chemical and electrophoretic analyses of the serum or plasma proteins of a part of the monkeys studied are given in Tables II and III. Table II summarizes data on control and Table III on vitamin-deficient monkeys. A serum pattern of a control and of a vit. B_6-deficient monkey are given in Fig. 1 and 2. The electrophoretic values of the individual components are given as relative concentrations which are expressed in percent of the total area. The electrophoretic mobilities of the plasma or serum components of a group of control and deficient monkeys are given in Table IV. The relative concentrations of the various components of serum or plasma vary considerably in individual control animals. However, the values are similar to those observed by Deutsch and Goodloe(1). The component "f" described by the latter has been found in the patterns of the majority of the monkeys studied and its presence or absence is indicated by (+) or (—) in the tables. It is possible that this component occurs in all monkey patterns but may have been obscured in a number of instances. An average value of 1% of the total proteins has been obtained for this fraction. This value is approximately twice that reported by Deutsch *et al.*

In the case of control monkeys maintained on diet S, the average electrophoretic mobilities of the gamma globulin and Ø are higher, and those of the remaining components are lower than the corresponding values reported by Deutsch and Goodloe(1). Since measurements of mobility are not in general extremely precise, no great significance is attached to these differences. The mobility of the "f" component was not calculated.

Effect of vit. B_6 deficiency. With the exception of one animal (No. 16), all monkeys

TABLE II. Chemical and Electrophoretic Data* on Serum and Plasma Proteins of Control Monkeys.

Serum (S) or Plasma (P)	No. of animals	Diet	Chemical: Total protein, g %	Albumin, g %	Globulin, g %	A/G	Electrophoretic, % of total area: Albumin	α 1 and 2	α 3	β	Ø	γ	A/G	f
P†	5	S	6.7 ± .2	3.9 ± .15	2.8 ± .15	1.4	49 ± 1.65	9.5 ± 1	7 ± .9	15 ± 1.5	11 ± .3	9.5 ± .6	.95	Present in 4 animals
S†	6	S	7.45 ± .2	4.4 ± .3	3.3 ± .2	1.3	57 ± 1.05	7.5 ± .6	8 ± 1.4	15 ± 1.35		13.5 ± 1	1.3	
S†	2	HP	6.7	4.35	2.35	1.85	56.7	13	9.25	12.25		11.25	1.3	Present in 2 animals
P	1	HP	6.6	4.1	2.5	1.6	47	11	16	8	9.5	8.5	.9	?
S	1 (#31)	AP	6.8	4.6	2.2	2.1	66	6	9	8.5	—	10.5	1.95	?
S	1 (#38)	MFC‡	7.9	4.85	3.05	1.6	50.5	8.5	15	10	—	14.5	1	
S	1 (#21)	SC‡	7.45	4.1	3.3	1.2	48	9.5	17	9	—	16.5	.9	

* Since α-1 and α-2 were not sharply separated in a few instances, the sum of the two are given throughout.
† Represent average values. ± standard error of mean. ‡ 1% cholesterol added to diets S and MF.

TABLE III. Chemical and Electrophoretic Data* on Serum or Plasma Proteins of Vitamin Deficient Monkeys.

Animal No.	Deficiency	Diet	Deficiency, mo	Serum (S) or plasma (P)	Chemical Total protein, g %	 Albumin, g %	 Globulin, g %	 A/G	Electrophoretic % of total area Albumin	 α 1 and α 2	 α 3	 β	 Ø	 γ	 f	 A/G	Liver damage
16	B6	S	28	S	6.6	4.15	2.45	1.7	55	15	5	8	—	17	?	1.2	
16	,,	S	32	S	6.95	3.75	3.2	1.15	48.5	7.5	10	12	—	22	+	.95	
54	,,	S	8½	S	4.1	2.2	1.9	1.2	44	5	3	21	—	27	?	.8	+
56	,,	S	11	S	6.4	1.9	4.5	.4	25	3	5	17	—	49	+	.35	+
56	,,	S	11½	S	4.7	1.3	3.4	.4	19	5	4	20	—	52	+	.2	+
35	Low B6	MF	a	S	6.7	3.4	3.3	1	52	13	9.5	7.5	—	18	+	1.1	
35	,,	MF	b	P	6.7	1.8	4.9	.35	36	9.5	14	8	18.5	14	+	.55	+
35	,,	MF	c	S	6.1	2.9	3.2	.9	47	8.5	15	11	—	19.5	?	.9	+
36	,,	MF–C†	d	S	7.2	3.5	3.7	.95	42.5	7.5	17	9.5	—	23.5	+	.75	
37	,,	MF–C†	e	S	7.5	3.8	3.7	1	42.5	9	29.5	12	—	12	+	.75	
37	,,	MF–C†	f	S	6	2.2	3.7	.6	25.5	9	28.5	20	—	22	?	.35	+
41	,,	S	g	S	7.45	4.05	3.4	1.2	48	10	8	13	—	21	+	.9	
41	,,	S	h	S	6.95	4.1	2.85	1.45	52	11	4.5	15.5	—	17	?	1.1	
42	,,	S	i	S	6.65	4.15	2.5	1.65	59	10.5	8	10.5	—	12	+	1.45	
42	,,	S	j	S	6.25	3.55	2.7	1.3	48	12.5	7	14.5	—	18	+	.9	+
43	,,	S	k	S	6.9	3.65	3.25	1.1	42	7.5	12	12.5	—	26	?	.7	
22	,,	S	l	S	7.8	3.75	4.05	.9	51	5	9	13.5	—	20.5	—	1.1	
25	,,	S–C†	m	S	7.5	3.9	3.7	1.05	52.5	9	12	13	—	14	+	1.1	
25	,,	S–C†	n	S	7.1	4	3.1	1.3	50	8.5	11.5	12.5	—	17.5	+	1	
26	Choline	AP	31½	S	6.15	3.5	2.65	1.3	45	12	6	16.5	—	20	—	.8	
27	,,	AP	34¼	S	7.05	3.4	3.65	.95	46.5	19.5	11	15.5	—	7.5	+	.85	
28	Niacin	AP	17½	S	7.45	4.6	2.85	1.6	58.5	7	5	9.5	—	20	+	1.4	
28	,,	AP	19	S	8	4.8	3.2	1.5	53.5	8.5	4	10.5	—	23.5	+	1.15	
29	,,	AP	13	S	8.15	5.2	3	1.75	57	7	8	11	—	16	+	1.3	
59	Asc. acid	S	o	P	7.4	3.4	4	.85	47.5	9.5	15	4.5	13.5	10	—	.9	
60	,,	S	p	P	7.1	3.8	3.3	1.15	40	14	7.5	13.5	10.5	15	—	.65	
61	,,	S	q	P	7.7	3.9	3.8	1	48.5	8.5	8.5	10.5	13	10.5	+	.95	
62	,,	S	r	P	7	3.3	3.7	.9	36	13.5	9	15	13.5	13	?	.55	

* Since α-1 and α-2 were not sharply separated in a few instances, the sum of the two are given throughout.

† See reference ‡ Table II.

a No B6 2 mo; 25 γ 5¼ mo
b 12
c 14
d 4.5
e 4.5
f 15
g 15

h No B6 2 mo; 25 γ 17 mo; followed by 1 mg 64 days
i 30 γ 15¼ mo
j 17¾
k 15¼

l 250 γ B6 9¼ mo; 125 γ 9¾ mo; 60 γ 15½ mo
m 24¾
n 25¾
o No ascorbic acid 2¼ mo; 1 mg 20 days
p 2¾ 11
q 2¼ 19
r 21

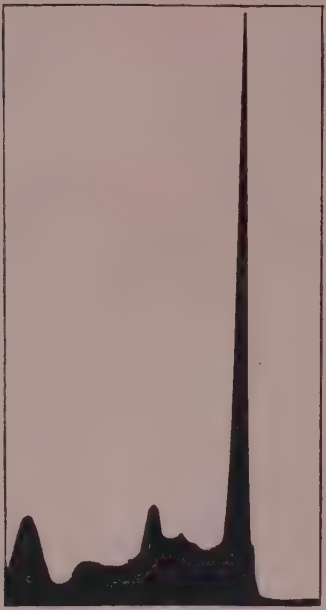


FIG. 1. Ascending pattern—control serum.

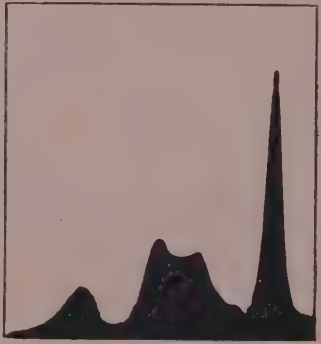


FIG. 2. Ascending pattern—vitamin B_6 deficient serum.

on diet S which were deprived of vitamin B_6 showed marked lowering of the albumin, but an increase in the gamma globulin concentration was observed in all. Lowered albumin and increased total globulin are also evident in the chemical data. Gamma globulin values as high as 52% have been observed. Since gamma globulin has been shown to be related to liver damage(12-15) and a number of these monkeys were found to have had marked liver injury, this was not entirely unanticipated. Deficient animals which have come to autopsy and in which liver damage was evident are indicated in the last column of Table III. A few animals also exhibited a significant increase in beta globulin. Significant increases in the gamma-globulin component were also evident in the majority of animals receiving a lowered intake of the vitamin. The mobilities of the serum components do not appear to be influenced by either partial or total deprivation of vit. B_6.

Effect of cholesterol feeding. Cholesterol feeding to control monkeys results in a significant increase in the alpha-3 component (Nos. 38 and 21). One animal receiving the high protein diet (HP) also exhibited an increase in this component. Increase in the beta component of serum of the rabbit as a result of cholesterol feeding has been reported by Fishberg *et al.*(16). Two animals (Nos. 36 and 37) subjected to a low intake of pyridoxine while on a moderate fat diet containing cholesterol showed, in addition to the increase in the alpha-3 component, a lowering of the albumin and an increase in the gamma globulin.

Low choline diet. Only 2 animals (Nos. 26 and 27) were followed on this diet (AP). In comparison with the one control animal (No. 31) on this diet, both exhibited significant decreases in albumin and increases in the beta-globulin components. In addition, monkey 26 was found to have an increased concentration of the gamma component, while No. 27 also showed a significant rise in the alpha-1 plus alpha-2 components.

Low niacin-low B_{12} diet. Although 2 animals were maintained on a diet lacking in both niacin and vit. B_{12} for a period of over 21 months these monkeys did not exhibit any obvious manifestation of either deficiency. These animals (Nos. 28 and 29) are listed in Table III merely as niacin deficient. In comparison with the serum of their control animal (No. 31) the sera of the 2 monkeys maintained on this diet were observed to have a lowered albumin and an increased concentration of gamma globulin.

Ascorbic acid deficiency. No significant alterations were observed in the serum protein patterns of 4 monkeys (Nos. 59 to 62) who

TABLE IV. Electrophoretic Mobilities of Serum or Plasma Proteins of Control and Vitamin Deficient Monkeys.

Animal No.	Deficiency (D) and control (C)	Duration, mo	Diet	Serum or plasma	Mobilities (CM^2/volt sec. × 10^{-5}) Albumin	α 1	α 2	α 3	β	Ø	γ
Avg (18, 40, 53, 34)	C		S	S	5.29	4.67	4.27	3.66	3		1.72
51	C		HP	S	5.1	4.5	4.05	3.5	2.9		1.5
21	C		S+chol.	S	6.2	5.4	5.05	4.25	3.7		2.5
38	C		MF+chol.	S	5.3	4.4	4.2	3.8	3.4		2.2
16	D-B6	32	S	S	5.2	4.5	4.3	3.6	3.1		1.75
50	D-B6	14	S	S	4.9	4.05	3.65	2.95	—		2.6
41	a		S	S	5.5	4.65	4.2	3.6	3.15		1.4
42	b		S	S	5	—	3.9	3.1	2.6		1.05
42	c		S	S	5.7	—	4.65	4	3.5		2.1
43	d		S	S	5.2	4.6	4.1	3.6	3		1.55
22	e		S	S	5.1	4.35	3.85	3.4	3		1.35
25	f		S+chol.	S	6.2	5.4	4.85	4.3	3.8		2.15
25	g		S+chol.	S	5.1	4.3	4.05	3.4	3		1.45
37	h		MF+chol.	S	5.9	—	5.15	4.5	3.5		1.75
26	D-choline	31½	AP	S	5.5	4.75	4.4	3.9	3.5		2.06
28	D-niacin	17½	AP	S	5.4	4.6	4.4	3.7	3.35		1.9
31	C		AP	S	5.15	4.25	4.1	3.4	2.75		1.5
57	C		S	P	4.9	4.3	3.85	3.7	2.8	2	1.35
58	C		S	P	5.25	4.55	4.1	3.6	3	2.35	2
Avg (57, 58)					5.07	4.42	3.97	3.65	2.9	2.17	1.67
59	i		S	P	5.9	5.25	4.7	4	3.35	—	2.3
60	j		S	P	5.7	5.05	4.55	3.9	3.5	2.6	1.8
61	k		S	P	6.25	—	5.35	4.45	4.15	3.25	2.35
62	l		S	P	6.15	5.6	5.3	4.45	4.05	3.3	2.75
		Avg			6	5.3	4.9	4.2	3.76	3.05	2.3

a No B6; 2 mo, 10 γ/kg 15 mo
b 15¼
c 17¾
d 15¼
e 250 γ B6, 9¼ mo; 125 γ B6, 9¾ mo; 60 γ B6, 15½ mo
f 24¾ mo
g Same as f except 125 γ B6 for 25¾ mo
h No B6; 2 mo, 10 γ/kg 15 mo
i D—Ascorbic acid 2¼ mo; 1 mg 20 days
j D— 11
k D— 19
l D— 21

were investigated during the early stages of chronic scurvy, *i.e.*, 2¼ to 2¾ months of complete deficiency followed by a daily supplement of 1 mg of ascorbic acid for 11 to 21 days. There appeared to be some increase in the mobility of the various components in the scorbutic animals (Table IV). However, this requires further study.

Discussion. Numerous publications(12-15) have reported hypoalbuminemia and hyperglobulinemia (primarily of the gamma component) as the characteristic alteration of the serum proteins in chronic liver disease. Since generally one observes various degrees of liver damage ranging from mild fatty infiltration to marked cirrhosis in the vit. B_6 deficient monkey, it is not surprising that hypoalbuminemia and marked hyper-gamma-globulinemia occur. It is quite possible that the hypoalbuminemia is the result of interference with albumin synthesis, since there is considerable evidence which indicates that vit. B_6 plays an important role in metabolism of proteins and amino acids. Previous experiments on malnutrition in humans(17,18) and in animals(19) have been offered as evidence (15) that malnutrition is not responsible for the rise in gamma globulin in chronic liver disease. These observations have dealt mainly with protein underfeeding or depletion. Since vit. B_6 deficiency in the monkey is a rather slowly progressing and debilitating disease and is similar in many respects to a state of chronic malnutrition, the former could be classified as a specific type of chronic malnutrition. On this basis the present work affords evidence that this specific type of malnutrition (vit. B_6 deficiency) may bring about a marked increase in gamma globulin. Some evidence is also reported which indicates that hypoalbuminemia and hyper-gammaglobulinemia may also occur in other vitamin deficiencies, such as niacin.

Summary. The plasma or serum proteins of rhesus monkeys maintained on various vitamin deficient diets were studied by chemical and by electrophoretic analysis. Partial or complete deficiency of vit. B_6 resulted in hypoalbuminemia and a significant increase in the gamma globulin component. On a niacin deficient diet 2 monkeys developed a lowered albumin and an increase in the gamma globulin. A low choline diet led to a decrease in the albumin and to an increase in the beta globulin. The addition of 1% cholesterol to the diet of control or vit. B_6 deficient monkeys produced a significant increase in the alpha-3 component. The electrophoretic composition of the plasma did not appear to be altered by a state of early chronic scurvy. The mobilities of the plasma components appeared to be increased by an ascorbic acid deficient diet but were unaffected by the other regimens.

We are grateful to Lederle & Co., Pearl River, N. Y., for supplies of Folacin and to Squibb and Co. for vit. B_{12}.

---

1. Deutsch, H. F., and Goodloe, M. B., *J. Biol. Chem.*, 1945, v161, 1.

2. Moore, D. H., *ibid.*, 1945, v161, 21.

3. Rinehart, J. F., Greenberg, L. D., and Ginzton, L. L., *Blood*, 1948, v3, 1453.

4. Reiner, M., and Fenichel, R. L., *Science*, 1948, v108, 164.

5. Longsworth, L. G., *Chem. Rev.*, 1942, v30, 323.

6. Ross, V., Moore, D. H., and Miller, E. G., Jr., *J. Biol. Chem.*, 1942, v144, 667.

7. Cohen, P. P., and Thompson, F. L., *J. Lab. and Clin. Med.*, 1948, v33, 75.

8. Longsworth, L. G., *J. Am. Chem. Soc.*, 1939, v61, 529.

9. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, v69, 119.

10. Wolfson, W. Q., Cohn, C., Calvary, E., and Ichiba, F., *Am. J. Clin. Path.*, 1948, v18, 723.

11. Reinhold, J. G., Bluemle, Jr., L. W., Campoy, F., Gilman, L., Gould, E. F., Martens, V. L., and Shuman, R., *Fed. Proc.*, 1949, v8, 242.

12. Gutman, A. B., *Advances in Protein Chemistry*, Academic Press, New York, 1948, v4, 155.

13. Rafsky, H. A., Weingarten, M., Krieger, C. I., Stern, K. G., and Newman, B., *Gastroenterol.*, 1950, v14, 29.

14. Ricketts, W. E., Sterling, K., Kirsner, J. B., and Palmer, W. L., *Gastroenterol.*, 1949, v13, 205.

15. Franklin, M., Bean, W. B., Paul, W. D., Routh, J. I., de la Huerga, J., and Popper, H., *J. Clin. Invest.*, 1951, v30, 718 and 729.

16. Fishberg, A. M., Friedfeld, L., Hoffman, I., Stroller, E. R., and Fishberg, E. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 301.

17. Keys, A., Taylor, H. L., Mickelson, O., and Henschel, A., *Science*, 1946, v103, 669.

18. Leutscher, J. A., Jr., *Physiol. Rev.*, 1947, v27, 621.

19. Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., *J. Exp. Med.*, 1945, v82, 157, 411.

Received January 29, 1952. P.S.E.B.M., 1952, v79.

Effects of Purine and Pyrimidine Analogues on Development of *Rana pipiens* (19402)

S. BIEBER, R. F. NIGRELLI, AND G. H. HITCHINGS.

From the Department of Biology, Washington Square College, New York University, The New York Zoological Society and The Wellcome Research Laboratories, Tuckahoe, N. Y.

Analogues of the purine and pyrimidine constituents of the nucleic acids have been found to have a variety of biological effects which may be ascribed to various types and degrees of interference with the biosynthesis of nucleic acids and related compounds(1).

The desirability of testing such antimetabolites on a variety of biological species and systems has previously been mentioned(1) for such studies contribute both to the comparative biochemistry of nucleic acid metabolism and to the knowledge of the mode of action of the antimetabolites. The present report is concerned primarily with an extension of the antimetabolite studies to the problems of chemical embryology. Although the effects

TABLE I. Inhibition of Development of *Rana pipiens* by Purine and Pyrimidine Analogues.

Substance	Earliest stage inhibited	Min effective conc., mg/100 ml
8-Azaadenine	8	7.5
6-Mercaptopurine	8–9	20
5-(2′, 4′-Dichlorophenoxy)-4-hydroxy-2-mercaptopyrimidine	8	5
2-Amino-5-(4′-chlorophenoxy)-4-hydroxypyrimidine	8–9	10
5-(4′-Chlorophenyl)-2,4-diaminopyrimidine	12–13	2.5
5-(4′-Chlorophenyl)-2,4-diamino-6-ethylpyrimidine	14–15	5
2,4-Diamino-5-(3′,4′-dichlorophenyl) pyrimidine	20	10
6-Amyl-5-(4′-chlorophenyl)-2,4-diaminopyrimidine	23	10
5-(4′-Chlorophenoxy)-2,4-diaminopyrimidine	13	10
5-(4′-Chlorophenoxy)-2,4-diamino-6-methylpyrimidine	14	10
2,4-Diamino-5-phenoxypyrimidine	16–17	10
5-(3′4′-Dichlorophenoxy)-2,4-diaminopyrimidine	21	10
5-Benzyl-2,4-diamino-6-methylpyrimidine	14–15	20
5-(4′-Chlorobenzyl)-2,4-diamino-6-methylpyrimidine	17–18	20
2,4-Diamino-5-(4′-nitrobenzyl)-6-methylpyrimidine	21	20
2,4-Diamino-5-(3′,4′-dimethoxybenzyl) pyrimidine	21	20
4-Amino-pteroylglutamic acid (Aminopterin)	12–13	25
8-Azaguanine	25+	10
5-Nitrouracil	25+	10
5-Bromouracil	25+	10
5-Hydroxyuracil	25+	10
2,4-Diamino-5,6-dimethylpyrimidine	23+	20
2,6-Diaminopurine	25+	50
Xanthopterin	*	50
2,4-Diamino-6,7-dimethylpteridine	*	15

* Not inhibitory at concentrations tested.

of some antimetabolites on the development of the chick have been studied, except for a brief attempt by Brachet(2) and a more recent preliminary report(3) amphibia have not been utilized for such studies. The amphibian egg lends itself readily to experimental and biochemical observations on early morphogenesis. The present investigation is concerned with the development of *Rana pipiens* embryos from fertilization to Shumway stage 25(4).

Methods. Eggs were obtained from female *Rana pipiens* by induced ovulation and were fertilized by a suspension of macerated frog testes in spring water. The twenty-five analogues were tested by immersing embryos chosen at the two cell stage in eight different concentrations of the compound (in the range of 1 to 25 mg per 100 ml) made up in spring water. Fifty embryos, 25 per finger bowl, were used for each concentration. The bowls were kept in an incubating refrigerator at 18-19°C during the course of the experiment. Readings of the developmental progress were taken at regular intervals and compared with those of appropriate controls.

The *results* are expressed in Table I in terms of the earliest stage of development which is inhibited by the analogue and the minimal effective concentration to produce complete inhibition at this point. At lower concentrations the development is inhibited at critical stages (8,9,12,13,15,16,20-23) but eventually passes through each stage, and between critical points development proceeds at essentially a normal rate even in the presence of the inhibitor. Thus certain substances which inhibit at an early stage may, at low concentrations, show retardation of development at several rather specific stages. In general it may be stated that the substances affecting the earliest stages of development are those which are found to affect purine metabolism primarily, in microbiological studies (5); those which inhibit at neurulation are, in general, antagonists of folic acid or citrovorum factor in the microbiological system (6,7). Embryos demonstrating inhibition at the later periods of development possess abnormalities such as reduction of head structures, failure to hatch, abnormal gill and opercular development, reduction in embryo size and other abnormalities indicative of interference with normal development.

The results of a more complete study under way(8), including histological, histochemical and reversal attempts, support the view that these effects reflect the interference with purine and pyrimidine utilization by the embryo rather than being due to generalized toxic reaction.

---

1. Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., Sherwood, M. B., and VanderWerff, H., *J. Biol. Chem.*, 1950, v183, 1.

2. Brachet, J., *Cold Spg. Hbr. Symp. Quant. Biol.*, 1947, v12, 18.

3. Rosenbaum, R. M., and Verlardo, J. T., *Nature*, 1951, v168, 424.

4. Shumway, W., *Anat. Rec.*, 1940, v78, 139.

5. Elion, G. B., Hitchings, G. H., and VanderWerff, H., *J. Biol. Chem.*, 1951 v192, 505.

6. Hitchings, G. H., Elion, G. B., VanderWerff, H., and Falco, E. A., *J. Biol. Chem.*, 1948, v174, 765.

7. Falco, E. A., Goodwin, L. G., Hitchings, G. H., Rollo, I. M., and Russell, P. B., *Brit. J. Pharm.*, 1951, v6, 185.

8. Bieber, S., Ph.D., Thesis, New York University, 1952 (Unpublished).

Received February 8, 1952. P.S.E.B.M., 1952, v79.

A Technic for Determination of Adrenocorticotrophin in Blood.* (19403)

KATHERINE L. SYDNOR AND GEORGE SAYERS.
(With technical assistance of Lurrine Burgess, Pearl Ofgreen, and Marva Jean Tobler.)
From the Department of Pharmacology, University of Utah College of Medicine, Salt Lake City, Utah.

The oxycellulose technic of Astwood *et al.* (1) for the isolation of adrenocorticotrophin (ACTH) from pituitary has been successfully adapted to the quantitative analysis of ACTH in rat blood.

Methods. Collection of blood. Male rats of the Sprague-Dawley strain, weighing 150 to 300 g, were employed. Each rat was anesthetized with ether and bled from the abdominal aorta into a heparinized syringe. Four volumes of glacial acetic acid were immediately added to the sample and the mixture was thoroughly stirred. Pools of blood-glacial acetic acid mixtures were processed for ACTH as described below.

Technic for concentration of blood ACTH. 1) The mixture of blood and glacial acetic acid is gently stirred at 70-75°C for 30 minutes in a water bath and then transferred to a large pyrex cylinder. 2) To each ml of the above mixture are added 13.0 ml distilled water with constant stirring. 3) 0.1 g Hyflo-Super-cel per ml of blood is stirred into the mixture which is then allowed to stand for 24 hours. The Super-cel settles to the bottom of the cylinder. 4) The supernatant is siphoned into a pyrex cylinder for further processing; the Super-cel is discarded. 5) A slurry of prepared† oxycellulose‡ (0.04 g per ml blood) is added to the supernatant with continuous stirring; stirring is continued for 24 hours to allow for complete "adsorption" of ACTH on oxycellulose. 6) The oxycellulose is now allowed to settle for 24 hours. 7) The supernatant is siphoned off and discarded; the oxycellulose is collected on a course, sintered-glass funnel and washed with 30-ml quantities of 0.1 N acetic acid until the washings are

---

* This investigation was supported by research grants from the American Cancer Society, recommended by the Committee of Growth of the National Research Council, and from the Medical Research and Development Board, Office of the Surgeon General, Department of the Army.

† A weighed amount of oxycellulose is washed into a sintered-glass funnel with three 25-ml quantities of 0.1 N acetic acid, then washed successively with 25 ml distilled water, 25 ml 0.1 N HCl, 25 ml distilled water and, finally, with three 25-ml quantities 0.1 N acetic acid. A slurry of the washed oxycellulose is prepared with 15 to 30 ml 0.1 N acetic acid.

‡ Samples of oxidized cellulose (11-12% COOH) were generously supplied by the Research Laboratories, Tennessee Eastman Co.

colorless. 8) ACTH is now eluted from the oxycellulose with 0.1 N HCl. The oxycellulose is washed into a beaker with an appropriate volume of 0.1 N HCl. The slurry is stirred frequently for one hour and then filtered by gravity or by gentle suction on the sintered-glass funnel. Two additional elutions are carried out and the eluates are combined. The minimum volume of 0.1 N HCl to make a slurry of workable consistency in the first elution is 15 ml per 4 g of oxycellulose. The volume of 0.1 N HCl used in the second and third elutions is determined by the dose to be injected into the assay rat. For example, if 100 ml blood are processed and the oxycellulose is eluted initially with 15 ml and then eluted twice with 5-ml quantities of 0.1 N HCl, each ml of combined eluate is equivalent to approximately 4 ml blood.

Method of assay. ACTH was assayed in hypophysectomized male rats by the adrenal ascorbic acid-depletion method previously reported from this laboratory(2). Unknown samples were compared with freshly prepared solutions of U.S.P. Standard ACTH in every assay. Results are expressed in U.S.P. milliunits (mu) of ACTH. One mu is one thousandth of a U.S.P. Unit of ACTH which is defined as the activity of one mg of the International Standard ACTH (La-1-A).

Results. Hypophysectomized rat blood. Three separate pools of blood obtained from rats hypophysectomized 24 hours prior to bleeding were assayed for ACTH. No significant ascorbic acid depletion occurred when a dose§ of eluate equivalent to 18 ml hypophysectomized rat blood was administered to the test animal. (Table I, Assays 1 and 2.)

Recovery of exogenous ACTH from hypophysectomized rat blood. A pool of blood was collected from rats 24 hours after hypophysectomy. Fifty U.S.P. mu of Standard ACTH were added to the blood immediately prior to processing. The eluate was diluted with 0.01 N HCl so that the administered dose contained 1.0, 0.5, or 0.25 mu of added ACTH. The results (Table I, Assay 1) indicate that 82 ± 12% of the added hormone was recovered.

Recovery of endogenous ACTH from adrenalectomized rat blood. The work of Gemzell *et al.*(3) demonstrated that plasma from adrenalectomized rats has a high concentration of ACTH. Preliminary studies employing the oxycellulose technic confirmed this observation and indicated that it would be possible to analyze the blood of adrenalectomized rats for ACTH without concentration. A comparison has been made of the estimate of blood ACTH of adrenalectomized rats by direct transfusion with that obtained by the oxycellulose technic.

Oxycellulose technic. Male rats weighing 150 to 300 g were bilaterally adrenalectomized and given a solution of 0.9% sodium chloride to drink. One week after adrenalectomy blood was collected and processed as described above. The equivalents of 12, 6, 3 and 0.75 ml of blood were administered to the test animals. The results of 2 assays (No. 2 and No. 3) indicate that each 100 ml of blood from these adrenalectomized rats contains 12.0 ± 2.5 mu and 10.3 ± 2.0 mu of ACTH, respectively.

Transfusion. Male rats, bilaterally adrenalectomized and maintained as described above, served as donors. In these experiments blood was not pooled; each test animal received blood from a single adrenalectomized donor. Immediately after the sample was obtained, a dose of 1.5, 3, 5 or 6 ml was injected via the saphenous vein of the hypophysectomized test animal. The results (Table I, Assays No. 3, 4 and No. 5) indicate that each 100 ml of blood from adrenalectomized rats contains about 15 mu of ACTH. Comparison of the value obtained by direct transfusion with that obtained by the oxycellulose technic suggests that the method recovers at least 70% of blood ACTH. The fact that transfusion of a dose of 6 ml of hypophysectomized rat blood into the test animal (Assay 5) induced no depletion of adrenal ascorbic acid eliminates the possibility that a large volume of blood *per se* produces a false positive response in the method of assay for ACTH.

Recovery of endogenous ACTH from intact rat blood. Two separate pools of blood were

---

§ Doses represent quantity administered per 100 g body weight of assay rat.

TABLE I. Assay Blood ACTH.

Assay No.	Sample	Dose/100 g assay rat	Response, mg ascorbic acid/100 g adrenal		Estimate of ACTH recovery or potency with S.E.
			Individual	Avg	
1	U.S.P. Std ACTH	1 mu	94, 132, 178, 114, 164, 139, 102, 138	133	
		.5	72, 131, 56, 129, 117, 119, 146	110	
		.25	36, 21, 52, 34, 77, 83, 84, 35	53	
	Hypox blood #1 (4 g oxycel./100 ml blood)	18 ml	–16, –15, –9	–13	0 mu/100 ml*
	Hypox blood #2 (4 g oxycel./100 ml blood)	18	26, –2, 0	8	
	Hypox blood + ACTH (4 g oxycel./100 ml blood)	1 mu	133, 131, 136, 112, 86, 114, 134, 147	129	82 ± 12% recovery
		.5	48, 97, 63, 112, 64, 93	86	
		.25	70, 99, 20, 38, 21, 58, 28, 67	50	
2	U.S.P. Std ACTH	1	148, 204, 128, 194, 148, 207	172	
		.5	47, 135, 106, 170, 129, 93	113	
		.25	6, 27, 20, 45, 88	37	
	Adrenex blood (4 g oxycel. /100 ml blood)	12 ml	177, 148, 166, 186	169	12±2.5 mu/100 ml
		3	54, 97, 72, 67, 129	84	
		.75	23, 12, 12, 57, –36, 20, 47	19	
	Hypox blood (4 g oxycel./ 100 ml blood)	18	–8, 5, 22, 26	11	0 mu/100 ml*
3	U.S.P. Std ACTH	1 mu	199, 212, 127, 255, 160, 171, 173, 157	182	
		.5	163, 195, 99, 187, 111, 169, 117, 144	148	
		.25	63, 79, 49, 26, 84, 99, 114, 121	79	
	Adrenex blood (4 g oxycel. /100 ml blood)	6 ml	216, 138, 190, 150, 203, 195, 119	173	10.3±2 mu/100 ml
		3	126, 166, 135, 127, 109, 147, 55	123	
	Adrenex blood (transfused)	5	159	159	8 to 17 mu/100 ml
		3	161, 69, 75, 90, 55, 138, 98, 102, 179, 149	112	
4	U.S.P. Std ACTH	1 mu	143, 156, 138, 133, 124, 127, 140, 155	140	
		.5	143, 100, 86, 107, 121, 95	109	
		.25	63, 36, 47, 32, 31, 35	41	
	Adrenex blood (transfused)	6 ml	99, 74, 123, 109, 144, 109, 182, 105, 198, 173	132	16.1±2.1 mu/100 ml
		3	96, 74, 129, 108, 95, 115, 103	103	
5	U.S.P. Std ACTH	1 mu	204, 104, 175, 79, 138, 185, 151	148	
		.5	105, 138, 161, 126, 86, 145	127	
		.25	90, 138, 135, 55, 77, 37, 77	87	
	Hypox blood (transfused)	6 ml	–41, 1, –34, 17, 1	–11	0 mu/100 ml*
	Adrenex ” ”	6	127, 165, 105, 163, 170, 103, 127	137	14±4.3 mu/100 ml
		3	113, 82, 114, 138, 101	110	
		1.5	50, 110, 91, 140	98	
6	U.S.P. Std ACTH	1 mu	68, 163, 159, 98, 176	133	
		.25	36, 87, 46, 47, 62, 56	56	
	Intact blood #1 (4 g oxycel./100 ml blood)	12 ml	60, 60, 67, 56, 58	60	2.1 ± .6 mu/100 ml
		6	19, 17, 1, 16, –12	7	
	Intact blood #2 (4 g oxycel./100 ml blood)	12	103, 82, 86, 52	81	2.9 ± .8 mu/100 ml
		6	15, 43, 47, 3, 15	25	
7	U.S.P. Std ACTH	1 mu	195, 118, 145, 112, 140, 110	137	
		.5	71, 123, 60, 82, 149	97	
		.25	25, 19, 35, 25, 33	27	
	Adrenex blood (2 g oxycel. /100 ml blood)	6 ml	72, 124, 56, 140	98	11.1 ± 2.2 mu/100 ml
		3	105, 59, 70, 73, 21	66	
	Same but .2 g	6	24, 17, 54, 11, 4, 19	22	< 25% recovery*†
		3	–31, –4, –24, –37	–24	
	Same but .02 g	6	25, 12, –1, –50, 0, 39	4	< 25% recovery*†
		3	–7, –23, –20, –23, 5, –16	–14	

* Estimate made without analysis of error.

† Assuming 2 g oxycellulose recovers 100%.

collected and processed. The eluate of each pool was administered in doses equivalent to 6 and 12 ml of blood. The results are recorded in Table I, Assay 6. The responses obtained for the two pools are in excellent agreement. Statistical analysis indicates that intact rat blood obtained by acute exsanguination contains 2 to 3 mu ACTH per 100 ml.

Recovery of endogenous ACTH from adrenalectomized rat blood with various quantities of oxycellulose. In the experiments reported above, 4.0 g oxycellulose were added to each 100 ml blood. The results of Assay 7 indicate that 2.0 g oxycellulose per 100 ml blood are as effective as 4.0 g in recovering blood ACTH (*cf.* potency adrenalectomized rat blood as determined by Assays 2 and 3 with potency as determined by Assay 7). However, quantities of 0.2 and 0.02 g are obviously insufficient.

Discussion. The adrenal ascorbic acid-depletion technic is not sensitive enough unequivocally to detect ACTH in unconcentrated blood of normal man or rat. Taylor *et al.*(4) did not find any significant depletion of adrenal ascorbic acid of the hypophysectomized rat after the administration of 2 to 3 ml of serum from normal man and from patients with Cushing's syndrome. In this laboratory (Richards)(5), 4 to 6 ml of blood obtained from the abdominal aorta of the intact rat failed to induce a significant depletion of adrenal ascorbic acid. Taylor *et al.*(4) have presented data which suggest that 2 to 3 ml of serum from individuals with adrenocortical insufficiency contain a detectable quantity of ACTH. It is apparent that biological activity must be concentrated in order to determine with accuracy the quantity of ACTH in blood. Cooke *et al.*(6) and Bornstein and Trewhella(7) have described technics for concentration of biological activity in plasma. However, they did not present experimental data to indicate the specificity or the quantitative recovery to be expected from these methods. Gemzell *et al.*(3) have claimed, without presentation of data, that an acid-acetone technic will quantitatively recover ACTH from rat plasma. In this laboratory, the acid-acetone technic has not been reproducible and has not yielded quantitative recovery of blood ACTH. Moreover, toxicity of acid-acetone concentrates of plasma and, especially, of blood is a decided disadvantage of this method.

The oxycellulose technic as described above satisfies the following criteria for a sound method for the determination of ACTH in blood. It appears to be *specific for ACTH,* as demonstrated by the fact that the blood of hypophysectomized animals induces no depletion of adrenal ascorbic acid in the assay rat. Experimental studies presented above indicate that the method *almost completely recovers ACTH added to blood.* The concentration of ACTH in the blood of the adrenalectomized rat, as determined by the oxycellulose technic, was nearly the same as that determined by transfusion of adrenalectomized rat blood directly into the test animal. These results suggest that the oxycellulose method *almost completely recovers endogenous ACTH.* The eluates have caused *no toxic reactions* in any instance. Limited experience to date suggests that the method is *highly reproducible* and is *applicable to other species* (dog and man¶).

No experimental data are available to justify all the steps enumerated under the description of technic. The technic as described is essentially that employed by Astwood *et al.*(1) for the isolation of ACTH from pituitary. Future developmental studies may make it possible to simplify the procedure for the analysis of blood ACTH. Without reliable information regarding stability of ACTH in blood it was deemed advisable to mix the sample with glacial acetic acid immediately after collection. As a routine procedure, the glacial acetic acid-blood mixture is diluted with water within four hours after collection of the sample.

Blood rather than plasma has been employed for two reasons. First, no information is available on the partition of ACTH between cellular elements and plasma. Second, the time required to separate cells and plasma may be sufficient for inactivation of blood ACTH.

¶ Preliminary studies indicate that the blood of patients with Addison's disease has a higher than normal concentration of ACTH.

The experimental results reported here are not strictly comparable with those reported by Gemzell *et al.*(3) since the latter analyzed blood from the inferior vena cava. Blood from the abdominal aorta was employed in the present studies. We have confirmed Gemzell *et al.*(3) that blood of the adrenalectomized rat has a greater concentration of ACTH than normal. However, these investigators were unable to detect activity in 448 ml of plasma from intact rats, whereas our data indicate that 12 ml of blood from these animals induce a definite depletion of adrenal ascorbic acid.

Summary. 1. The oxycellulose technic for isolation of ACTH from pituitary has been successfully employed to concentrate and quantitatively to measure blood ACTH. The method a) appears to be specific for ACTH since hypophysectomized rat blood induces no adrenal ascorbic acid depletion in the assay animal, b) almost completely recovers ACTH added to hypophysectomized rat blood, c) almost completely recovers endogenous ACTH, d) yields concentrates which are not toxic to the assay animal and e) is reproducible. 2. It is estimated that rat blood, obtained by acute exsanguination of intact animals, contains 2 to 3 mu ACTH per 100 ml, whereas blood obtained under similar conditions from rats bilaterally adrenalectomized for one week contains 10 to 12 mu ACTH per 100 ml.

---

1. Astwood, E. B., Raben, M. S., Payne, R. W., and Grady, A. B., *J. Am. Chem. Soc.*, 1951, v75, 2929.
2. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, v42, 379.
3. Gemzell, C. A., Van Dyke, D. C., Tobias, C. A., and Evans, H. M., *Endocrinology*, 1951, v49, 325.
4. Taylor, A. B., Albert, A., and Sprague, R. G., *Endocrinology*, 1949, v45, 335.
5. Richards, J., unpublished data.
6. Cooke, D. S., Graetzer, E., and Reiss, M., *J. Endocrinol.*, 1947, v5, lxxxix.
7. Bornstein, J., and Trewhella, P., *Lancet*, 1950, v2, 678.

Received February 8, 1952. P.S.E.B.M., 1952, v79.

A New Operative Preparation for Production of Peptic Ulcer in the Dog.* (19404)

LESTER R. SAUVAGE,† EVERETT J. SCHMITZ, EDWARD H. STORER, FRANKLIN P. SMITH, EDMUND KANAR, AND HENRY N. HARKINS.

From the Department of Surgery, University of Washington School of Medicine, Seattle.

Many data have been obtained by previous investigators concerning experimentally induced acid-peptic ulceration of the stomach and juxtapositioned parts of the alimentary canal. However, the methods employed to produce the ulceration are open to serious question. The procedure of duodenal drainage (Mann and Williamson) (1), causes severe nutritional difficulties in the dog leading to inanition. In addition, it has been shown (Storer, Oberhelman, Woodward, and Dragstedt) (2), that gastric secretion is greatly increased by this procedure. We feel that these objections have been satisfactorily overcome by our preparations to be described below. In the human and in the dog most of the secretion of the parietal cells occurs in response to neurogenic (vagal) or hormonal stimulation, the latter arising mainly from the antral mucosa and to a lesser extent from the small intestine. Accordingly, it is not physiologic to evaluate experimentally vagotomy or antrum resection in the dog when an exogenous stimulus to the parietal cells, as histamine, is employed.

We have devised an alternate method of producing experimental peptic ulceration in

---

* This work was aided in part by grants from the National Institutes of Health, Bethesda, Md., and Initiative 171 Research Funds of the State of Washington.

† Damon Runyon Fellow, 1950-2.

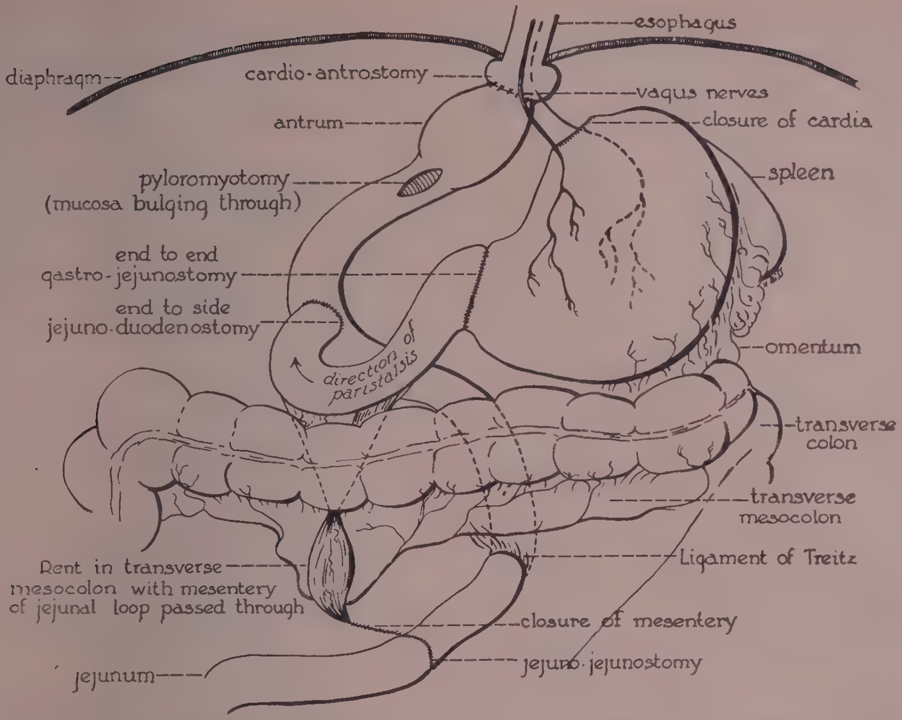


FIG. 1. A 3-phase total parietal cell pouch drained by a peristaltic jejunal fistula into the duodenum. Each of the 3 phases of gastric secretion (vagal, antral, and intestinal) are operating upon the pouch.

the dog and have studied the incidence of ulceration and average survival time in our animals. This method satisfies the following criteria for an acceptable ulcer producing preparation: 1. It must be a method by which ulcers can be produced (preferably rapidly) that will parallel as closely as possible peptic ulceration in man without requiring complicated post-operative care or artificial stimulation in the test animals. 2. It must permit evaluation of therapeutic methods as to their effectiveness in preventing peptic ulcers in these preparations. 3. When ulcer formation has been prevented (by some test procedure), the experimental animal must survive in good condition for prolonged periods of time.

Experimental methods. This preparation consists of a total parietal cell pouch drained by an iso-peristaltic jejunal fistula into the second portion of the duodenum. The procedure has been performed on healthy adult mongrel dogs through a left subcostal incision with the dog under nembutal anesthesia. The pouch is formed by dividing the stomach 1 cm distal to the esophagogastric junction and again at the upper border of the antrum. The proximal end of the pouch is closed with a running serosal suture. A jejunal fistula 15 cm in length is procured by transecting the jejunum at 5 and 20 cm distal to the ligament of Treitz. Jejunal continuity is reestablished by end-to-end anastomosis. The fistula is then made ante-colic or retro-colic and anastomosed end-to-end to the distal pouch and end-to-side to the duodenum. In the 3-phase total parietal cell pouch the vagi are preserved to the pouch and the antrum is anastomosed to the remnant of the cardia attached to the esophagus (Fig. 1). In the antral-intestinal phase pouch the vagi are divided and the antrum anastomosed to the cardia as in the 3-phase pouch. In the cephalic-intestinal phase

TABLE I. Influence of Vagotomy, of Antrumectomy, and the Two Combined upon Development of Ulcers in the Dog with a Total Parietal Cell Pouch Drained by a Peristaltic Jejunal Fistula into the Duodenum.

	3-phase pouch (cephalic, antral, intestinal)			2-phase pouch (antral, intestinal)			2-phase pouch (cephalic, intestinal)			1-phase pouch (intestinal)		
	No.	% of total	Avg survival, days	No.	% of total	Avg survival, days	No.	% of total	Avg survival, days	No.	% of total	Avg survival, days
Total dogs	16	100		10	100		9	100		11	100	
Dogs still living*	1	6	136	2	20	82	5	56	56	5	45	87
Dogs dead without ulcer†	0	0	0	0	0	0	1	11	106	5	45	37
Dogs dead with ulcer												
Gastric	3	19	9	0	0	0	0	0	0	1	9	33
Jejunal	9	56	26	8	80	32	3	33	34	0	0	0
Gastric and jejunal	3	19	21	0	0	0	0	0	0	0	0	0
Total dogs dead with ulcer	15	94	21	8	80	32	3	33	34	1	9	33

* Includes dogs living 4 weeks or more after operation.
† ” ” dying ” ” ” ” ” ” without ulcer.

pouch the vagi are preserved, but the antrum is excised and the cardia anastomosed end-to-end to the first portion of the duodenum. In the one-phase pouch the antrum is excised and the vagi divided; the cardia is anastomosed to the duodenum as in the cephalic-intestinal phase pouch. The esophagogastric sphincteric mechanism is preserved by leaving a small segment of cardia (usually less than 1 cm) attached to the esophagus. Animals in whom this was not done frequently suffered from continuous post-operative vomiting. Likewise in those dogs with the antrum in continuity pyloromyotomy greatly reduced post-operative vomiting. Retention of food was enhanced by an operative approach via the abdomen rather than through the chest. The division of diaphragmatic fibers about the lower esophagus apparently destroyed at least a part of the normal sphincteric mechanism. Post-operative care of these animals has consisted of subcutaneous fluids on the day of operation, penicillin for 3 days after operation, water as desired, milk on the 3rd day, soft food on the 4th day, and then general kennel rations as tolerated.

Results. Vomiting has not been a serious problem after the dogs have become adjusted to eating slowly. The 5 one-phase dogs that are still living an average of 87 days after the operation have lost an average of 10% of body weight. Their stools appear quite normal. Most of our dogs dying of causes other than ulcer have died of intestinal obstruction due to herniation of the small bowel behind the mesentery of the jejunal fistula that was placed in an ante-colic position. We now make all fistulas retro-colic.

Both fistulous and pouch ulcers have been observed, occasionally multiple and in some instances both types in the same animal. The jejunal ulcers typically have occurred just distal to the gastrojejunostomy and have varied in size from discrete ulcers 1 cm in diameter to large ulcerations extending the entire length of the fistula. Death usually resulted from generalized peritonitis subsequent to ulcer perforation. These ulcers have been of an acute nature on the basis of gross pathologic examination when the animal survived only a few days and of a more chronic nature when the animal survived for longer periods. The incidence of ulceration and the average survival times of the dogs in the 4 groups are shown in Table I.

Discussion. It appears that dogs are less severely impaired nutritionally by this procedure than by the Mann-Williamson procedure. That the 3-phase pouch is a potent ulcerogenic preparation is attested to by the fact that 94% of these dogs died with ulcer an average of 21 days after operation. Our preliminary results would seem to indicate that antrumectomy in the dog affords greater protection against the development of ulceration than does vagotomy. Antrumectomy and vagotomy combined appear to give the dog a considerable measure of protection against the development of ulceration. This method of producing peptic ulceration in the dog may have certain advantages over the standard methods that will render it useful for further experimental study of the ulcer problem.

Summary. 1. A method for production of experimental peptic ulcer in dogs is presented. Its advantages are reliability, simplicity of post-operative care, rapidity of ulcer development, and survival of animals in good nutritional condition, thus allowing prolonged study in protected animals. The ulcers are produced without extraneous stimulation. 2. Four groups of animals were studied. The effect of vagotomy alone, antrumectomy alone, and vagotomy combined with antrumectomy are evaluated as to incidence of ulcer and period of survival (an indication of the time required for development of ulcer).

---

1. Mann, F. C., and Williamson, C. S., *Ann. Surg.*, 1923, v77, 409.

2. Storer, E. H., Oberhelman, H. A., Woodward, E. R., and Dragstedt, L. R., in press.

Received February 9, 1952. P.S.E.B.M., 1952, v79.

Cation Adsorption by Alginic Acid in Humans.* (19405)

Harold S. Feldman, Karl Urbach, Charles F. Naegele, Frederic D. Regan,
and Alexander A. Doerner.

From the Department of Medicine, U.S.P.H.S. Hospital, Staten Island, New York.

The removal of exogenous sodium from the intestinal tract and prevention of its absorption into the body by means of cation exchange resins has offered a new therapeutic approach to the management of the edematous state. In clinical practice, however, the use of these agents has been attended by several undesirable effects(1), including unpalatibility, anorexia, fullness, vomiting, constipation and the potential hazard of sodium depletion and acidosis. The search for substances with similar clinical promise, but without the limitations and disadvantages of the resins has led to the investigation of alginic acid. It is the purpose of this report to describe initial experiences and record laboratory data on the cation adsorbing ability of this agent in humans.

Alginic acid† is a water-insoluble amorphous solid with marked hydrophilic properties which is widely used in the food and pharmaceutical industry as a stabilizing, emulsifying and thickening agent. Chemically the substance is a polymer of anhydro-B-D-mannuronic acid. It is extracted from giant kelp (*Macrocystis pyrifera*) and is closely related to cellulose and pectic acid. Each anhydromannuronic acid residue contains one free carboxyl group which is responsible for its theoretical cation adsorption capacity of 5.7 meq. per g. *In vitro* studies(2) have shown that sodium adsorption by alginic acid is primarily dependent on hydrogen ion concentration of the medium. In unbuffered solutions of sodium chloride, alginic acid combined with less than 1 meq. sodium/g and this was not appreciably influenced by changes in sodium concentration. During the adsorption process liberation of hydrogen ion lowered the pH of the medium from neutrality to approximately 3. However, when equilibrated with sodium ion in solutions buffered to approximately physiological pH of 7.6 the sodium adsorption per gram of alginic acid rose to 4.2 meq. These *in vitro* studies suggested that alginic acid had cation adsorption capacities similar to synthetic resins. Feeding experiments in animals indicated the non-toxicity of alginic acid derivatives(3).

---

* The authors are indebted to the Dietetic Department of the U. S. P. H. S. Hospital, Staten Island, N. Y. for the help necessary to carry out this work.

† Supplied through the courtesy of Wallace Laboratories, New Brunswick, N. J.

Material and methods. Three patients were selected for this study whose clinical condition warranted sodium restriction. Patient C.K. was a 56-year-old white male with severe cirrhosis of the liver and ascites. Patient N.P. was a 50-year-old white male with hypertensive cardiovascular disease, class III C (N. Y. Heart Association classification). Patient C.N. was a 59-year-old white male with arteriosclerotic heart disease and chronic left heart failure, class IV, D. A fourth patient was selected as a control. This patient, N.Z. was a 35-year-old white male with no illness other than a Meniere's syndrome. Full cooperation of the patients was secured and detailed studies were carried out under controlled conditions in a special unit of the Medical Service. Following a 7-day control period during which time each patient received a diet calculated to contain approximately 500 mg of sodium per day, this dietary regimen was complemented by the addition of 15 g of alginic acid 3 times daily for 7 days. In 3 of these patients this experiment was repeated utilizing a diet containing approximately 1500 mg of sodium per day. Total 24-hour urine volumes were measured daily, aliquots were taken and sodium and potassium analysis of the pooled specimens was carried out every 3 or 4 days with a Perkin-Elmer flame photometer. Similarly, all stool specimens were pooled every 3 or 4 days and sodium and potassium determinations were done according to the procedure of Eisenmenger(4). Most of the determinations were performed in duplicate and occasional cross checks were made on the same specimen by means of two different flame photometers with different sets of standards. The greatest difference in any 2 determinations was less than 5%. Other observations included daily body weights, and plasma electrolytes and electrocardiograms taken at weekly intervals. The patients were examined daily for presence of edema and their subjective reaction to the medication was noted.

Results. Plasma electrolytes and serial electrocardiograms showed no appreciable changes during the entire experimental period. All four patients tolerated the alginic acid well when taken stirred up in water. However, during the first 2 days of medication, complaints of a transient, slightly unpleasant taste were noted, as well as occasional brief sensations of fullness. In no instance did anorexia, nausea or vomiting occur. A mild but effective laxative action of the drug was observed in all patients. The data on sodium and potassium excretion in feces and urine for each control and medication period, as well as stool weights and urine volumes are presented in Table I. All data are recorded as daily averages calculated on the basis of seven-day pooled specimens.

Discussion. The data presented clearly indicate that in every instance except one, the daily average excretion of fecal sodium increased with alginic acid medication. The fecal potassium excretion increased in every instance during the administration of alginic acid. These increases in fecal sodium and potassium excretion occurred even in those cases in which total sodium excretion dropped during the alginic acid period as contrasted with the control period. It would thus seem probable that this increased fecal excretion of sodium and potassium was due to the alginic acid medication.

The discrepancy noted in those cases in which the total sodium excretion dropped on alginic acid in comparison with the control period may be attributed to variations in dietary sodium intake. Although the diets were carefully calculated from accepted standard tables(5), a random sample diet calculated to contain 10 meq. of sodium proved on actual analysis to contain 48 meq. of sodium.

Statistical analysis shows the increase in daily fecal sodium and potassium excretion to be significant (p smaller than 0.05 for Na, and smaller than 0.01 for K). Although daily stool weights increased in every instance on alginic acid medication, these increases were not found to be statistically significant.

The body weights showed only minor variation throughout the period of study except in patient C.K., who had a marked diuresis, which was at least partially attributable to the

TABLE I.

Patient	Diet Na	Urine vol, ml/day		Urine Na, meq/day		Urine K, meq/day		Feces wt, g/day		Fecal Na, meq/day		Fecal K, meq/day	
		O	A	O	A	O	A	O	A	O	A	O	A
K.	500	638	1077	29.5	22.6	37.5	37.9	81	151	1.5	5.4	7.4	14.9
	1500	1071	1424	21.6	57.7	46.1	50.3	102	164	2.2	10.2	5	14.6
P.	500	2307	2332	87.1	48.7	82.1	51.8	166	161	4.9	2.2	21.7	27.4
	1500	2560	2237	96.3	88.5	101.1	87.7	151	248	2.2	9.2	19.5	36
N.	500	1489	2017	89.8	48.4	102.6	65.9	76	135	1.9	2.9	7.9	21.8
Z.*	500	2074	2252	53.2	24.7	74	45.5	119	185	3	6.1	10.6	12.7
	1500	1727	2050	69.1	38.2	72.7	50.9	233	349	6.7	15.8	26.3	43.4

* Z (control patient).
O = control period; A = alginic acid period.

fact that his regular mercurial medication was temporarily increased. Despite this, his fecal excretion of sodium significantly increased. The large increase in urinary sodium excretion in this instance was felt to be associated with the marked diuresis of this cirrhotic patient.

The results of this study indicate that the adsorption of sodium by alginic acid is approximately one-fifth to one-sixth of the values reported for cation exchange resins in clinical use(6). The reasons for the discrepancy between the *in vitro* cation adsorption capacity of alginic acid and the results reported here are not clear. It may be that partial digestion of alginic acid occurs, as has been reported in animals(3), or that an insoluble calcium alginate is formed in the stomach.

In view of the definite sodium adsorbing capacity of alginic acid, although admittedly small in comparison with cation exchange resins, and because of its favorable tolerance by patients and lack of marked undesirable side effects, it is felt that further trial and clinical investigation may be warranted. Possible chemical modification of alginic acid to increase its *in vivo* adsorption capacity may be worthy of study.

Conclusions. 1. Alginic acid is well tolerated in humans when ingested in quantities of 45 g per day and is mildly laxative. 2. Alginic acid increases fecal sodium and potassium excretion in humans to the extent of approximately one-fifth to one-sixth that of the cation exchange resins in clinical use.

1. Danowski, T. S., Greenman, L., *et al., Ann. Int. Med.,* 1951, v35, 529.

2. Ludwig, B. J., Holfeld, W. T. and Berger, F. M., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 176.

3. Nilsen, Hugo W., Wagner, John A., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 630.

4. Eisenmenger, William J., *et al., J. Clin. Invest.,* 1950, v29, 1941.

5. Martz, B. L., Helmer, O. M., and Kohlstaedt, K. G., *Proc. Central Soc. Research,* 1950, v23, 70.

6. Sodium and Potassium Analysis of Foods and Waters, 5th List, Oct. 1947, Mead, Johnson and Co.

Received February 11, 1952. P.S.E.B.M., 1952, v79.

Vitamin E in Pituitary Gland Function of Fowls. (19406)

E. H. HERRICK, IRENE M. EIDE, AND MILTON R. SNOW.
(Introduced by G. K. L. Underbjerg.)
*From the Department of Zoology, Kansas State College.**

The necessity of vit. E for proper function of the anterior pituitary gland has been a controversial problem for a number of years. Verzar(1), in early studies, stated that vit. E acted like anterior hypophyseal hormone in inducing precocious sexual maturity in young rats, and that vit. E might be necessary for the formation of hypophyseal hormone. Diakov and Krizenecky(2) and Mattill(3) believed that vit. E did not exhibit gonadotropic effects, and that chorionic gonadotropin and corpus luteum extracts did not maintain the testes of rats on an E-free diet. Drummond, Noble, and Wright(4) found that vit. E had no effect on the reproductive organs of immature or hypophysectomized rats. They also concluded that hypophysectomy of vit. E-deficient rats caused further decline in weight of the testes and that the pituitary glands of the E-deficient rats contained an increased amount of gonadotropic hormone. The appearance of castration cells in the pituitaries of animals on an E-free diet is of particular significance. Mason(5), Evans and Burr(6), Nelson(7), and Mattill(3) found castrate cells in the pituitary glands of male rats that had been on an E-free diet for 120 days. They did not, however, find castration cells in female rats that were on an E-free diet. Severinghaus(8) stated that castration cells do not develop in the pituitary glands of guinea pigs while on a diet deficient in vit. E. The work of Van Wagenen(9), Nelson(7), and Mattill(3) disclosed, however, that castration cells in the pituitary developed during a deficiency in vit. E. Rowland and Singer (10) concluded that when there is a deficiency of vit. E, the pituitary gland does not have the capacity to produce an ovulation-producing substance which will cause ovulation in the oestrus rabbit. Müller and Müller(11) kept female rats on a vit. E-free diet for 137-240 days with no morphological changes in the pituitary gland. After 280 days on the diet, however, the pituitaries showed castration cells. Stein(12) found no notable pituitary changes from an E-free diet in rats. Nelson(7) found that vit. E is necessary for normal pituitary and testis growth. Biddulph and Meyer(13) found that after rats had been on a diet deficient in vit. E the testes showed reduction of about 50% in size but that pituitary glands from these animals were not altered in gonadotropic potency. They further report an increase in size of the basophilic cells after the animals had been on the E-deficient diet for some 6 months.

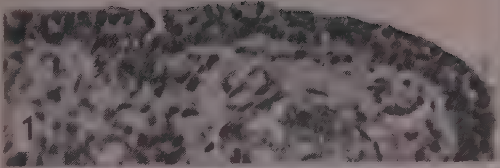
---

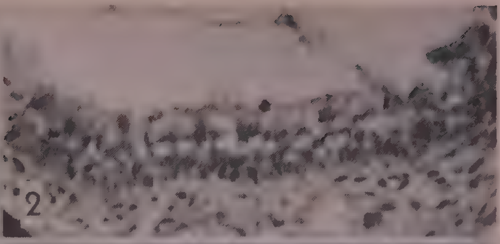
*Contribution No. 238, Serial No. 281, Department of Zoology, Kansas Agric. Exper. Station, Manhattan.

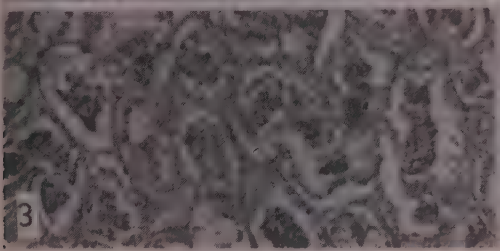
Prior to 1944 a series of 4 experiments were conducted in this laboratory to test the gonadotropic activity of the pituitary glands of chickens that were on a diet deficient in vit. E. An abstract was published(14). Recently this work was repeated but the results were so uniform in all groups of animals and both series of tests, the data are combined in those places in which experimental conditions were parallel.

Materials and methods. In 6 different groups 164 male chickens, one-half of which received vit. E and the other half deprived of vit. E, were studied. They were 6 weeks of age when the E-free diet was started and they were kept on it for 8 weeks. The basic diet used in all experiments consisted of: dry casein 18%, salt mixture 4%, corn starch 36%, rice 20%, dried brewers yeast 10%, lard 5%, fiber (ground cane fiber or ground oat hulls) 5%, and fish oil extracts 2% (or equivalent levels of vit. A and D). To one-half of the birds, vit. E was supplied in the form of mixed tocopherols. The diets were available to the birds at all times. At the termination of each experiment the birds were killed and their pituitary glands removed. The pituitary glands from 21 of the birds that received vit. E and a like number that did not receive the vit. E, were fixed in Heidenhain's Susa fixative for histological study. All others from both groups were prepared for the assay of gonadotropic hormones.

Observations. The pituitary glands prepared for histological study were stained with Heidenhain's Azan triple stain, a modification of Mallory's triple stain. Glands from the birds fed an E-free diet showed many basophils that were similar to those described by Severinghaus(8) as being in the later stages of secretion. This is also in agreement with results described by Barrie(15). The nuclei were highly pyknotic and the cytoplasm was much reduced in quantity. Counts of types of cells indicated that the glands from birds supplied with vit. E contained close to 100% more active basophils than those from birds given no vit. E. No castrate cells were found in the birds deprived of vit. E. In these experiments the birds were on the E-free diet a







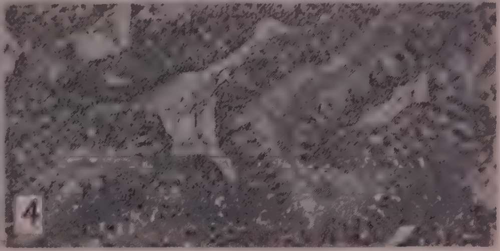


FIG. 1. Photograph of section of vaginal epithelium from assay rat that had received pituitary substance from animals deficient in vit. E.

FIG. 2. Photograph of section of vaginal epithelium from assay rat that had received pituitary substance from animals supplied with vit. E.

FIG. 3. Photograph of section of testis tissue from chicken that had received no vit. E for 8 weeks.

FIG. 4. Photograph of section of testis tissue from chicken fed the same diet as in Fig. 3, except that vit. E had been added to the diet.

shorter period of time than that reported by others in which castrate cells were observed. In the experiments reported here, however, the testes showed marked degenerative changes (Fig. 3).

In the birds that received vit. E the testes appeared normal in size and structure and averaged 9.71 g each in weight (Fig. 4). The thickness of the walls of the seminiferous tubules measured 44.29 μ. In the birds deprived of vit. E, the testes averaged 6.56 g in weight and the walls of the seminiferous tubules measured 27.10 μ in thickness. The relative degree of activity of the testes was reflected in the size of the combs. In 2 experimental groups that were weighed, the combs of the E-fed group weighed 44 g, whereas those from the E-deficient group averaged 20 g each.

Procedure and results of assay. All pituitary glands that were removed for assay purposes were ground to a paste and then combined with distilled water, one ml for each gland. The suspension was injected into sexually immature female mice or rats once daily for 3 days, a total of 0.3 ml being given at each injection. Following the series of injections the test animals were killed and the vaginal tracts were removed, fixed and sectioned. As a means of evaluating the degree of gonadotropic activity the thickness of the vaginal epithelium was measured. Care was exercised to choose regions of the vagina for measurement that were at the same distance from the cervix and four measurements were made for each test animal. The mean of the measurements from the E-fed groups, in which rats were used for the assay, was 58.25 μ whereas the mean of the E-deficient groups was 38.78 μ (Fig. 1 and 2). An analysis of all measurements within the groups (Snedecor, p. 80, 16) reveals a statistically significant difference between these two means ($t = 5.21$ and $P = <.001$). The measurements in which mice were used as the assay animals were not incorporated here since they were in a different range of magnitude although their comparative values were essentially the same as those reported here for the rats. It is concluded from these results that pituitary glands of male chickens contain more gonadotropic hormone if vit. E is supplied in the diet than if it is not supplied.

Summary. The testes of male chickens kept on a diet deficient in vit. E for two months were much reduced in size, and the seminiferous tubules showed considerable degeneration. The combs, likewise, were smaller in the group deprived of vit. E. The assays of pituitary glands revealed that more gonad stimulating substance was produced by those of fowls which had received vit. E in their diet. The pituitary glands from the birds on the E-deficient diet had approximately one-half as many active basophilic cells as those from the birds with the E supplied.

Acknowledgement is made to Quaker Oats Co. for supplying ground oat hulls and to Distillation Products, and Ayerst, McKenna and Harrison for supplying the tocopherols.

---

1. Verzar, F., *Pfluger's Arch.*, 1931, v227, 449.

2. Diakov, F. A. and Krizenecky, J., PROC. SOC. EXP. BIOL. AND MED., 1933, v31, 58.

3. Mattill, H. A., *J. Am. Med. Assn.*, 1938, v110, 1831.

4. Drummond, J. C., Noble, R. L., and Wright, M. D., *J. Endocrinology*, 1939, v1, 275.

5. Mason, Karl E., *J. Exp. Zool.*, 1926, v45, 159.

6. Evans, H. M. and Burr, George O., *J. Biol. Chem.*, 1928, v76, 293.

7. Nelson, Warren O., *Anat. Rec.*, 1933, v56, 241.

8. Severinghaus, Aura E., *Assn. Res. Nerv. and Mental Dis.*, 1936, v17, 69.

9. Van Wagenen, Gertrude, *Anat. Rec.*, 1925, v29, 398.

10. Rowland, I. W. and Singer, E., *J. Physiol.*, v86, 323.

11. Müller, J. R. and Müller, C., *Endokrinologie*, 1933, v118, 369.

12. Stein, S. I., *J. Nutr.*, 1935, v9, 611.

13. Biddulph, Clyde and Meyer, R. K., *J. Physiol.*, 1940, v132, 259.

14. Herrick, E. H., *Anat. Rec.*, 1944, v89, 537.

15. Barrie, M. M. O., *Lancet*, 1937, v223, 251.

16. Snedecor, G. W., *Ames, Iowa State Col. Press*, 1946.

Received February 15, 1952. P.S.E.B.M., 1952, v79.

Pulmonary Infarction from Cardiac Catheterization.* (19407)

HECTOR E. J. HOUSSAY,† FLORENCE W. HAYNES, AND LEWIS DEXTER.
(Introduced by S. J. Gray.)

From the Peter Bent Brigham Hospital and the Department of Medicine, Harvard Medical School.

The major complications that have been reported as a result of cardiac catheterization have been reviewed by Zimdahl(1) and have been of two general types: First are a variety of cardiac arrhythmias developing largely as a result of the introduction of the catheter into the right auricle and ventricle. These have consisted of ventricular premature beats, ventricular tachycardia, ventricular flutter, ventricular fibrillation, right bundle branch block, and heart block. Auricular premature beats, auricular flutter, paroxysmal auricular tachycardia, and auricular fibrillation also have occurred when the tip of the catheter has been in the right auricle. The second type of complication has been thrombosis occurring in the veins at the venotomy site and sometimes propagating centrally. In one reported case (2), in whom the catheter was introduced into the heart by way of a leg vein, a thrombosis propagating centrally to the heart resulted in death several weeks later.

We wish to report the production of pulmonary thrombosis and infarction in a series of patients with pulmonary congestion in whom a branch of the pulmonary artery has been occluded in order to record the pulmonary "capillary" pressure.

Methods. To measure the pulmonary "capillary" pressure, the catheter is introduced by the usual fashion into the pulmonary artery and then advanced further until it is wedged into one of the distal branches of this vessel(3). On the basis of about 100 experiences with this procedure, it was reported (3) that no untoward reactions occurred. In March of 1950, however, the first of a series of pulmonary infarctions began to occur in patients with mitral stenosis and congestive heart failure following cardiac catheterization. At first, these infarctions occurred only sporadically, but later occurred with increasing frequency. Between March and November of 1950, only 4 such infarctions occurred, 2 in patients with congestive heart failure and 2 in patients with mitral stenosis. Between November, 1950 and May, 1951, pulmonary infarcts were produced in 15 of 27 patients with mitral stenosis. In no instance did death occur, but many of the patients had a stormy course for several days. Had it not been for the necessity of catheterization to determine the patients' operability, this procedure would hardly have been justified. There was a striking absence of this complication in patients with many types of congenital heart disease, pulmonary disease with cor pulmonale, various types of heart disease without pulmonary congestive phenomena, and liver disease.

---

* This work was supported by grants from the Life Insurance Medical Research Fund and the National Heart Institute, U. S. Public Health Service.

† Fellow of the Rockefeller Foundation.

Observations. Infarction was recognized in these cases by the occurrence of a rise of temperature, pulse, and respiration in all cases, associated with malaise, and often cough, signs of consolidation in the affected area, frank hemoptysis, blood-streaked sputum, pleuritic pain, friction rib, and a shadow in the lung by x-ray. With one exception, the infarct occurred in the area plugged by the catheter, from which it was concluded that this represented thrombosis *in situ* rather than embolism. In the one case in whom the infarct occurred at a distant site, it was presumed to have been on the basis of embolism from a right auricular thrombus. The symptoms and signs of infarction occurred as early as two and a half hours after wedging the pulmonary artery with the catheter and as late as 79 hours.

Age did not appear to be a factor, since infarction occurred in one patient who was only 14 years old. The company manufacturing the catheters[‡] had not changed the surfacing lacquer(4). Three factors, however, appeared to be mainly concerned with the production of this complication in these patients. 1) Only in patients with pulmonary congestion did pulmonary infarction occur. Venous thrombosis in the legs and pulmonary infarction from embolism are particularly prone to occur in patients with congestive heart failure(5,6), although a definite abnormality of the clotting mechanism in these patients has never, to our knowledge, been established. This thrombophyllic tendency presumably varies considerably from patient to patient and represents fertile soil for the development of infarction. 2) The peak incidence of pulmonary infarction occurred in these patients at a time when the greatest effort was being made to obtain as good a "capillary" pressure tracing as possible. The catheter was left *in situ* for longer periods of time than previously, and the pressure was often checked in several different areas of the lung. Careful washing of the surface and lumen of the catheter with saline and attempts to shorten the period of wedging of the pulmonary artery to an irreducible minimum, however, did not prevent pulmonary infarction. Even full heparinization of the patients before wedging the catheter did not eliminate infarction. 3) During the period when infarctions were produced, the catheters were sterilized by soaking in a 1:1000 solution of mercurioxycyanide. In our original publication(3), they had been sterilized by autoclaving. On the reinstitution of sterilization by autoclaving in May, 1951, pulmonary infarction following catheterization abruptly ceased. There have been no infarcts in 20 consecutive patients with severe mitral stenosis or congestive heart failure studied since that time.

Other factors such as the length of time the catheter was left in place, variation in the roughness of the surface of the catheter and in the degree of traumatization of the vessel wall by the catheter, and the care with which the surface of the catheter was washed with saline before its introduction into the vein presumably played an aggravating role. There seems to be no doubt, however, that the *sine*

[‡] U. S. Catheter and Instrument Company, Glens Falls, N. Y.

qua non in the production of infarction has been the chemical sterilization of the catheters.

Summary. 1. Pulmonary infarction has occurred in 19 patients with severe mitral stenosis or congestive heart failure 2½ to 79 hours following wedging of a cardiac catheter into a branch of the pulmonary artery for the measurement of pulmonary "capillary" pressure. 2. Over a period of 6 months, pulmonary infarction occurred in over half the patients studied with severe mitral stenosis. 3. In other patients without pulmonary congestion, pulmonary infarction did not occur. 4. When the catheters were sterilized by autoclaving instead of by soaking in mercurioxycyanide, pulmonary infarction did not occur in 20 consecutive patients with either severe mitral stenosis or congestive heart failure.

---

1. Zimdahl, W. T., *Am. Heart J.*, 1951, v41, 204.
2. Johnson, A. L., Woolin, D. G., and Ross, J. B., *Canad. M. A. J.*, 1947, v56, 249.
3. Hellems, H. K., Haynes, F. W., and Dexter, L., *J. Applied Physiol.*, 1949, v2, 24.
4. Jeckel, C. M., Personal communication.
5. Dexter, L., *Med. Record and Ann.*, 1950, v44.
6. Allen, A. W., Linton, R. R., and Donaldson, G. A., *Ann. Surg.*, 1943, v118, 728.

Received February 15, 1952. P.S.E.B.M., 1952, v79.

Hemolysis in Hydrogen Peroxide of Erythrocytes of Premature Infants. Effect of Alpha-tocopherol.* (19408)

HARRY H. GORDON† AND JAMES P. DE METRY.

From the Department of Pediatrics, University of Colorado School of Medicine.

György and Rose reported that administration of tocopherol prevented intravascular hemolysis and hemoglobinuria in vit. E deficient rats injected with alloxan(1). They found, also, that the red blood cells of rats deficient in tocopherol could be completely hemolyzed, while those of tocopherol-treated animals were never affected by incubation with dialuric acid or alloxantin, reduction products of alloxan(2). Since *in vitro* hemolysis was more marked with dialuric acid than with alloxantin, the authors suggested that dialuric acid might be the active moiety. Because Owens and Owens(3) had suggested that a dietary deficiency of vit. E might be responsible for retrolental fibroplasia, the erythrocytes of 14 premature infants, both with and without the disease, were tested for sensitivity to dialuric acid, using the procedure of Rose and György(4). The infants showed no evidence of E deficiency as measured by this test(5). Many premature infants do not absorb fat well, and Wright, Filer, and Mason (6) have shown that low serum tocopherol levels develop in these infants when fed diets designed to circumvent the handicap in fat absorption. Since the manifestations of E deficiency vary in different species, and in the same species at different ages(7), the possibility remained that E deficiency might exist in premature infants even though the dialuric acid test did not so indicate. The finding by György, Cogan, and Rose(8) that the red blood cells of newborn full term infants, resistant to dialuric acid, were hemolyzed by dilute solutions of hydrogen peroxide, and that this hemolysis could be prevented by alpha-tocopherol led to the present study of prematurely born infants. A significant degree of hemolysis has been found and this is consistently prevented by the feeding of alpha-tocopherol.

Subjects and methods. Observations were made in 23 premature infants, born either at the Colorado General Hospital, or admitted to its Premature Infant Center because of inadequate facilities for care at the place of birth. The infants' weights ranged from 745

---

* Aided by a Grant from the Association for the Aid of Crippled Children.

† Present address: Sinai Hospital of Baltimore, Baltimore, Md.

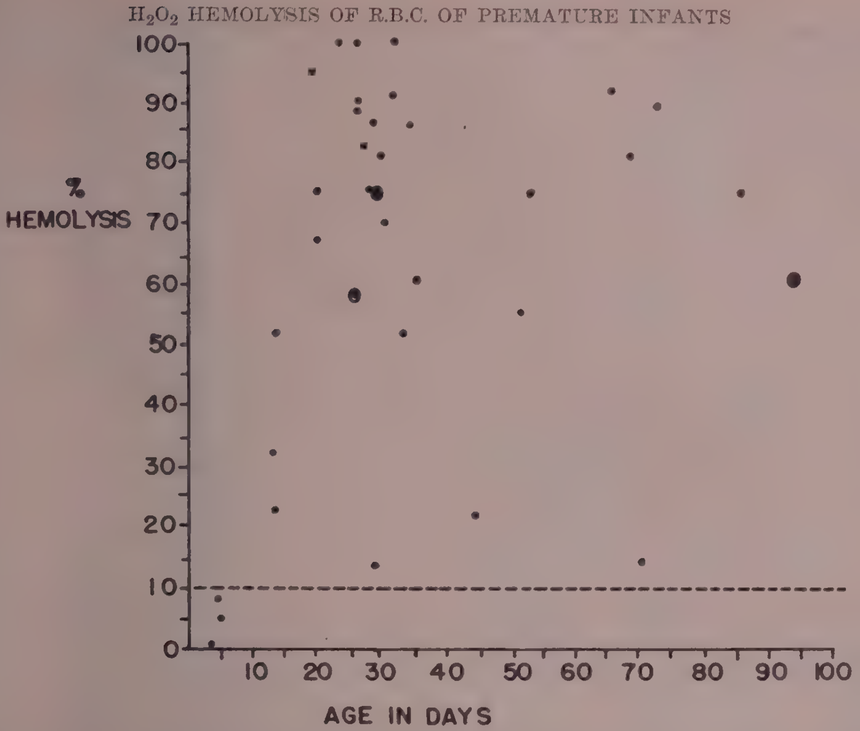


FIG. 1. Three infants, indicated by heavy dots, were fed evaporated milk. All others received partially skimmed milk. Dotted line indicates level below which all 19 observations on adults were found.

to 2000 g at the time of birth, and from 1100 to 3400 g at the time of testing. Their diets consisted, except as indicated, of a powdered preparation of partially skimmed cows' milk (Alacta) diluted with water and fortified with added carbohydrate. All but 5 infants had received supplements of ascorbic acid, 50 mg, and of vit. D, 1000 units, (Drisdol, 4 drops), daily, for 1 to 6 weeks before testing. One infant received ascorbic acid, 1.0 g in 4 divided doses on one day, for study purposes. Vit. E was given to 8 infants, either as an emulsion of alpha-tocopherol acetate, kindly supplied by the Hoffmann-La Roche Co., or as water-miscible tocopherol, Esorb (Wyeth). The hemolysing action of H_2O_2 was also tested on blood taken from 14 healthy adults, all professional hospital personnel, and 5 hospital patients.

Methods. Two-tenths ml of blood was drawn, usually from the antecubital vein, into a syringe previously rinsed with sterile saline-citrate solution, and transferred to a tube containing 2.0 ml of saline-citrate. The test was performed according to directions kindly supplied by Drs. György and Rose, with one modification, *i.e.,* 0.25 ml of 2.4% hydrogen peroxide was used, instead of 0.05 ml of 12% peroxide. This step made it possible to obtain, in duplicate or triplicate, checks within ±5%. All determinations reported herein were within this range, with the exception of 5, which were within ±6-9%. Samples of blood, from an adult and from both non-supplemented and tocopherol fed infants were generally tested simultaneously. Percent of hemolysis is calculated from the ratio of the color of the supernatant fluid in tubes containing the blood cells exposed to H_2O_2 to that in tubes in which complete hemolysis was obtained in distilled water. Readings were made at 550 μ in a Coleman spectrophotometer.

Results. Thirty-two observations in 20 infants on the diet of partially skimmed milk, and single observations in each of 3 infants who had been fed an evaporated milk mixture for 8, 10, and 30 days prior to observation are presented in Fig. 1. It is seen that, in all but 8 of the observations, hemolysis in hydrogen peroxide was more than 50% of that in distilled water. Of these 8, only 3 showed less than 10% hemolysis, the level below which all the observations on the 19 adults fell. A full-term infant of 18 months with pellagra and tuberculosis also showed only 5% hemolysis.

Each of 3 premature infants whose erythrocytes showed resistance to H_2O_2 was 4 or 5 days old, and 2 of them who were followed (Subjects J and M(F)) developed increased susceptibility to H_2O_2 with increasing age (Table I). It is planned to study more newborn premature infants to permit comparison with the data of György and his coworkers on newborn full-term infants. Of 2 full-term infants studied at age 12 hours, one showed 11%, and one 47% hemolysis. The mother of the latter infant showed 2% hemolysis.

In Table I are presented data on 9 infants on whom multiple observations were made. The effect of tocopherol administration was determined in 8 of them. It is seen that in every one of 9 trials in the 8 infants, there was a prompt drop in the hemolysis to the levels found for adults. The smallest dose tried was 25 mg daily for 2 days (Subjects 4 and 9); hemolysis dropped from 83 and 91% to 10% in Subject 4, and from 89 and 72% to 0 in Subject 9. In one infant, Subject 7, the administration of 25 mg of tocopherol daily for 4 days caused a drop from 100 to 4%, with a return to 91% hemolysis on the 10th day after discontinuing tocopherol supplements. Susceptibility to H_2O_2 persisted for another 7 days, when the administration of tocopherol again reversed the test. In infant No. 8, on the other hand, the administration of 25 mg of tocopherol for 5 days was followed by a persistently negative test as long as 22 days after discontinuing tocopherol. In infant No. 9, inconsistent results were obtained after discontinuing tocopherol. The effect of the administration of tocopherol on

TABLE I. Hydrogen Peroxide Hemolysis of Erythrocytes of 9 Premature Infants—Effect of Tocopherol Administration.

Subject	Birth wt, g	Age, days	Wt, g	% hemolysis*
J. ♀	1150	4	1100	8
		13	1200	22
		20	1440	67
		28	1680	76
M. ♀	1460	5	1390	5
		19	1640	95
		26	1740	100
		27	As 1 g	
		28	1825	87
		33	1925	100
		34–41	B 50 mg daily	
		38	2240	6
		41	2240	3
M. ♂	1250	52	1460	75
		66	1790	93
		67–80	C 150 mg daily	
		73	2000	0
		80	2470	0
E. ♂	2040	27	2070	83
		32	2280	91
		32–33	C 25 mg daily	
		34	2360	10
A. ♀	1275	13	1370	52
		20	1470	75
		34	1870	86
		35–40	C 50 mg daily	
		42	2030	11
B. ♀	1475	13	1470	33
		14–27	C 150 mg daily	
		20	1580	0
		27	1720	0
C. ♂	1520	23	1650	100
		24–27	C 25 mg daily	
		29	1800	4
		37	2100	91
		39	2100	73
		44	2300	99
		45–52	B 50 mg daily	
		49	2450	2
		52	2610	1
G.A. ♂	1500	26	1790	90
		27–32	C 25 mg daily	
		33	1880	0
		42	2100	9
		52	2520	14
		55	2600	2
G.B. ♂	1440	26	1490	89
		31	1590	72
		31–32	C 25 mg daily	
		33	1600	0
		40	1820	90
		42	1800	13
		47	1950	68
		52	2080	12
		55	2370	21

* Hemolysis in H_2O_2 as compared with hemolysis in distilled H_2O.

As = Ascorbic acid. B = α-tocopherol (Esorb, Wyeth). C = Tocopherol acetate emulsion (Hoffmann-La Roche).

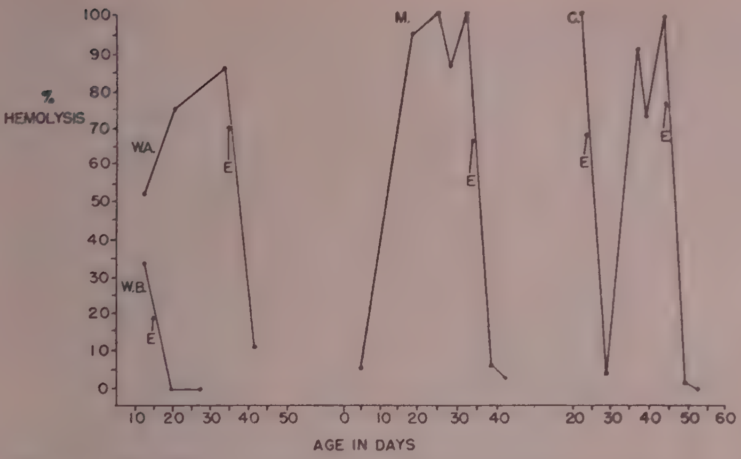


FIG. 2. Effect of α-tocopherol administration on H_2O_2 hemolysis of RBC of 4 premature infants. See Table I for dosage. Infants W. A. and W. B. were twins.

the hemolysis test in 4 of the infants is presented graphically in Fig. 2.

Discussion. The results of these studies confirm the findings of György, Cogan, and Rose(8) that susceptibility of red blood cells to dilute hydrogen peroxide can be prevented by administration of alpha-tocopherol to young infants. The effect of other substances on susceptibility to H_2O_2 remains to be studied; neither the administration of vit. D in doses of approximately 1000 units daily or ascorbic acid, 50 mg daily, prevented its development. Since ascorbic acid has been reported, along with glutathione and cysteine, as being capable of producing the *in vitro* hemolysis of the red blood cells of E-deficient rats(9), the role of ascorbic acid, a customary dietary supplement, will require investigation.

Wright, Filer, and Mason(6) have suggested, on the basis of studies of serum tocopherol levels, that the diets of premature infants should perhaps be supplemented with vit. E. They point out that premature infants may have special needs for tocopherol because of their rapid rate of growth, and the low content of tocopherol of the partially-skimmed milk mixtures widely used for their feeding. According to the data of Quaife (10), Harris(11), and Wright(6), it has been estimated that these partially-skimmed feeding mixtures contain approximately 0.04 mg of tocopherol per 100 cc, as compared with 0.10 mg % in customary evaporated milk mixtures, and 0.15 mg % in "mature" human milk. At an intake of 150-200 cc/kg/day, the tocopherol intake of a premature infant would be approximately 0.08 mg/kg as the one-half skim milk mixture, 0.2 mg/kg as evaporated milk, and 0.3 mg/kg as human milk. In 3 infants who had received an estimated 0.2 mg/kg tocopherol daily as evaporated milk for 8, 10, and 30 days before testing (Fig. 1), the tests showed hemolysis of 57, 61, and 75%. Whether a total intake of 0.3 mg/kg as human milk by premature infants would be adequate to prevent the susceptibility to hydrogen peroxide, in the light of their known defect in fat absorption, remains to be determined.

There is no evidence as yet that the susceptibility of the red blood cells of premature infants to hydrogen peroxide, and its consistent reversal by alpha-tocopherol, have physiologic or clinical significance. If this can be proven, however, then the data herein reported would support the regular addition of tocopherol to the diets of premature infants. The occurrence of edematous states during life and of hemorrhagic transudations terminally in these infants is reminiscent of the exudative diathesis reported in E deficient chicks(12,13), and the hemorrhagic state in the E deficient fetus of the rat(14).

Christensen and Dam(15) have reported

that dietary methylene blue which protects E deficient chicks against the exudative diathesis and brown coloration of adipose tissue also affords E deficient rats a marked, though not complete, protection against hemolysis of erythrocytes in dialuric acid. Antioxidants, other than alpha-tocopherol, may also be able to reverse the susceptibility of the red blood cells of premature infants to hydrogen peroxide.

Summary. Hemolysis of red blood cells in dilute hydrogen peroxide was determined for 23 premature infants. Three infants received evaporated cows' milk, and the remainder partially-skimmed cows' milk feeding mixtures. A significant degree of *in vitro* hemolysis was found and this persisted as long as the infants were observed without supplementation of their diets with tocopherol. In each of 9 trials in 8 infants the *in vitro* hemolysis was reversed by feeding alpha-tocopherol.

1. Gyorgy, P., and Rose, C. S., *Science,* 1948, v108, 716.
2. ———, *Ann. N. Y. Acad. Sci.,* 1949, v52, 231.
3. Owens, W. C., and Owens, E. U., *Am. J. Ophth.,* 1949, v32, 1631.
4. Rose, C. S., and Gyorgy, P., Proc. Soc. Exp. Biol. and Med., 1950, v74, 411.
5. Cohen, S., de Metry, J. P., and Herrmann, H., Proc. Soc. Exp. Biol. and Med., 1951, v77, 134.
6. Wright, S. W., Filer, L. J., Jr., and Mason, K. E., *Pediatrics,* 1951, v7, 386.
7. Mason, K., *Yale J. Biol. and Med.,* 1942, v14, 605.
8. Gyorgy, P., Cogan, G., and Rose, C. S., *Am. J. Dis. Child.,* 1951, v82, 237.
9. Rose, C. S., and Gyorgy, P., *Blood,* 1950, v5, 1062.
10. Quaife, Mary L., *J. Biol. Chem.,* 1947, v169, 513.
11. Harris, P. L., Quaife, Mary L., and Swanson, W. J., *J. Nutrition,* 1950, v40, 367.
12. Dam, H., and Glavind, J., *Skand. Arch. f. Phys.,* 1939, v82, 299.
13. Bird, H. B., and Culton, T. G., Proc. Soc. Exp. Biol. and Med., 1940, v44, 543.
14. Mason, K., *Essays in Biology,* Univ. Calif. Press, Berkeley and Los Angeles, p399, 1943.
15. Christensen, F., and Dam, H., *Acta pharmacol. et toxicol.,* 1951, v7, 167.

Received February 19, 1952. P.S.E.B.M., 1952, v79.

Dephosphorylation of Adenosine Triphosphate by Concentrates of the Virus of Avian Erythromyeloblastic Leucosis.* (19409)

E. B. Mommaerts, Edward A. Eckert, Dorothy Beard, D. G. Sharp, and J. W. Beard.

From the Department of Surgery, Duke University School of Medicine, Durham.

The virus of avian erythromyeloblastic leucosis(1) occurs in high concentration in the plasma of chicks affected with the disease. Concentration and purification of the agent have been effected(2,3) by the application of ultracentrifugal procedures to such plasma passed through earthen filters. The morphology of the virus and the characters of the concentrates obtained in this way have been described(3). In the course of studies of the chemical constitution and properties of the virus, inquiry was made into any possible activity which the virus might exert on adenosine triphosphate. It was learned, that the virus concentrates and other preparations containing the virus possessed the capacity to liberate inorganic phosphorus from this material. The importance of adenosine triphosphate as a source of metabolic energy(4) and the significance of its enzymatic dephosphorylation, possibly, by the virus in the absence of living tissue *in vitro* would appear to merit the present report of findings thus far encountered.

---

* This work was supported by research grants to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; and from the American Cancer Society on recommendation of the Committee on Growth; by a gift to Duke University from Lederle Laboratories Division, American Cyanamid Co.; and by the Dorothy Beard Research Fund.

Materials and methods. Experiments have been made with plasma from presumably normal chickens and from chicks diseased with erythromyeloblastic leucosis and with virus preparations obtained by ultracentrifugal fractionation of plasma from diseased birds. The character and origin of the strain of leucosis investigated have been described(5). All plasma was drawn into citrate, freed of cells by centrifugation, filtered through Celite and through Selas filters in the same way as that obtained for the purification processes. All preparations likely to contain free phosphorus were dialyzed against calcium-free Ringer solution in the cold. For *purification* of the virus, in a typical experiment, a pool of blood was collected by heart puncture into 6% sodium citrate in the proportion of one volume of citrate to 9 volumes of blood. The 53 donor birds with the disease, as diagnosed by blood smears(5), had been inoculated intravenously with infectious plasma 46 days previously at the age of 3 days and fasted for 24 hours before bleeding. The blood was centrifuged at 2,000 X g in 50 ml tubes in the horizontal centrifuge for 20 minutes for removal of the cells. The plasma was then recentrifuged under the same conditions, frozen in a dry ice-alcohol mixture and stored at about –14°C for 8 days. At the time of the experiment, the plasma was thawed in running tap water and centrifuged as described above to remove any clotted material. A 3 mm mat of Celite No. 512 was laid over a coarse filter paper, and 5 g of Celite No. 512 were added to each 120 ml of plasma. The plasma was filtered through the mat under suction and then passed through an 8″ Selas 02 filter (No. FP-88, Selas Corp. of America, Philadelphia, Pa.) under 4 lb of positive pressure. A volume of 420 ml of this clarified plasma was spun in the ultracentrifuge at 20,000 X g for one hour, and the resulting pellets were suspended in 42 ml of calcium-free Ringer solution containing 1% by volume of heparin (Liquaemin, 'Organon', 1 ml = 10 mg). The concentrate was centrifuged in the horizontal centrifuge at 2,000 X g for 10 minutes and the sediment discarded. The concentrate was spun at 20,000 X g for 30 minutes and resuspended in Ca-free Ringer solution containing heparin. This was repeated twice more for a total of 4 sedimentation cycles. Samples of each concentrate and supernatant fluid were taken for measurement of enzymatic activity and for nitrogen determination. The data obtained with each cycle are given in Table I.

TABLE I. Total Nitrogen Content and Dephosphorylating Activity on Adenosine Triphosphate of Plasma from Birds Diseased with Erythromyeloblastic Leucosis and of Ultracentrifugal Purification Fractions Derived from it in Experiment of Fig. 1.

		N/ml of reaction mixture, γ	Phosphate liberated/min/ml reaction mixture, μMol
Plasma pool		1775	.188
1st cycle	concentrate		.061
	supernatant	1900	.035
2nd cycle	concentrate	.77	.054
	supernatant	11.89	.011
3rd cycle	concentrate	.57	.038
	supernatant	.21	.004
4th cycle	concentrate	.50	.031
	supernatant	.04	.001

A study was made also with the purified virus preparation spun in an intermediate ultracentrifugal field. In this case, a concentrate obtained as described was placed in a 5 ml collodion tube, spun rapidly up to 10000 X g and held there for 3 minutes. The opalescent supernatant fluid was removed by pipetting. There was no real pellet at the bottom of the tube; instead, there was a small, somewhat opaque deposit on the wall. This was taken up in the volume of the concentrate initially used for this process. For the *measurement of enzymatic activity,* 1.8 ml of the virus preparation or plasma were mixed with 1.8 ml of adenosine triphosphate solution (adenosine triphosphate, disodium salt, Pabst Laboratories, Milwaukee, Wis.) brought to pH 7.2 with NaOH containing 3 mg of the salt per ml. Reaction took place at room temperature; at intervals 0.4 ml samples of the mixture were added to 4.6 ml of N perchloric acid. If necessary, the protein precipitate was removed by centrifugation. Phosphate determination was based on the method of Berenblum and Chain(6). The clarified

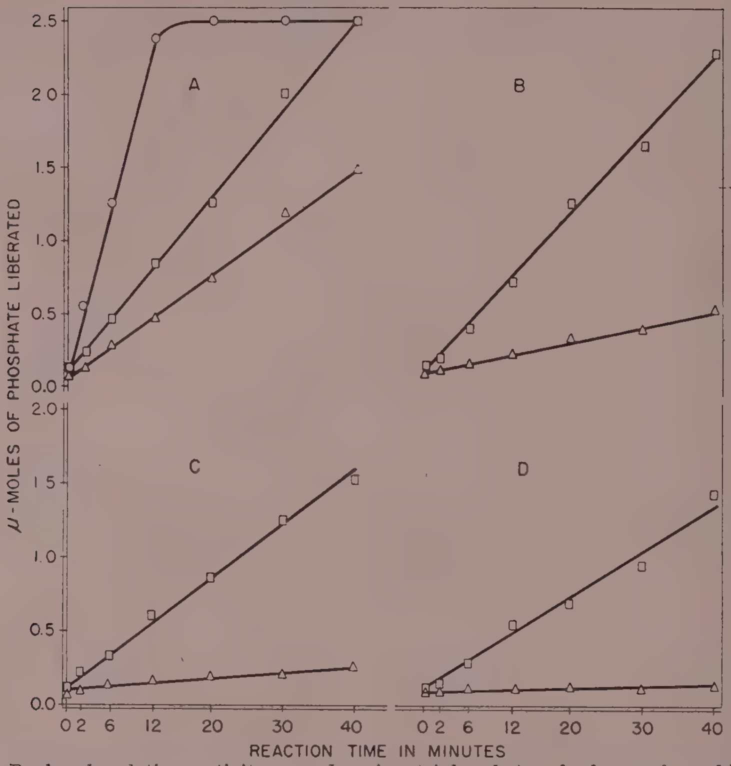


FIG. 1. Dephosphorylating activity on adenosine triphosphate of plasma from birds with erythromyeloblastic leucosis (circles of A) and of the concentrates (squares) and supernatant fluids (triangles) in 4 cycles (A, B, C, D) of sedimentation of the virus from this plasma.

sample was removed to a separatory funnel, followed by 2 ml of water washings, 2 ml of 5% ammonium molybdate, and 8 ml of isobutyl alcohol. The mixture was shaken for 1½ minutes, the aqueous layer was removed, and the alcohol layer was washed twice with 5 ml of N H_2SO_4, followed by 13 ml of stannous chloride solution (30 seconds shaking for each washing). The alcoholic solution was run into 10 ml volumetric flasks followed by the alcoholic wash fluid to volume. Absorption was read with a No. 600 filter in an Evelyn colorimeter.

Results. In Fig. 1 there is shown the character of the results obtained with the plasma from diseased birds and with the ultracentrifugal fractions resulting from the purification process through four sedimentation cycles. As seen in Fig. 1 A, the reaction with plasma was rapid, reaching completion in about 12 minutes. The pellet of the first sedimentation cycle resuspended in a volume proportional to that of the plasma was likewise exceedingly active, and the activity of the supernatant fluid of this cycle, the results with which are shown also in Fig. 1 A, was considerably less. Repetition of the sedimentation cycles resulted, Fig. 1 B, C, and D, in successive concentrates of relatively high, though progressively diminishing, activity, while all of the activity of the supernatant fluids was lost in the same procedures. This distribution of dephosphorylating activity in the concentrates and supernatant fluids closely parallels the partition of the infectious capacity of analogous fractions tested in repeated titrations in 3-day-old chicks. This is not meant to imply that the activity is associated only with infectious virus; instead, it is evident here that enzymatic activity is to be found in those fractions in which characteristic particulate material and virus activity reside. Consideration of the maximum amount of phosphorus freed at the equilibrium point indicates that, under the conditions of these experiments, only the third phosphorus is liberated with conversion of adenosine triphosphate to adenosine diphosphate.

The relation of nitrogen to enzymatic activity is shown in Table I. Here again it is evident that activity is associated with the particulate material rather than substances not susceptible to the centrifugal field employed. It is seen, also, that activity per unit of nitrogen in the concentrates is exceedingly high. Some of the activity of the original plasma was lost in the first sedimentation cycle, due in part to the considerable amount of material eliminated in the low speed centrifugation of the concentrate (see above) of this cycle.

Essentially identical results have been obtained with 7 different batches of plasma from chickens with erythromyeloblastic leucosis and 4 preparations of purified virus. Results such as those described above were obtained with freshly purified virus; experiments made daily on the same batch of the purified agent kept in the refrigerator at 2-4°C showed high activity for about 7 days, whereupon activity declined rather suddenly. This result is likewise compatible with the stability of the infectivity of this virus which declines rapidly even in plasma stored, frozen, at about −14°C.

Ultracentrifugation of the virus concentrate at 10000 X g, which would be expected to sediment a portion of the virus as well as masses larger than the virus, resulted in the elimination of about one-fourth to one-third of the enzymatic activity. Electron microscopy revealed only dispersed virus particles in the supernatant fluid. The sediment revealed an entirely different picture, containing not only some dispersed virus but many amorphous masses in which small numbers of virus particles seemed incorporated.

Experiments made with 4 different batches of plasma from normal chickens showed no evidence of enzymatic activity.

Discussion. The present experiments have revealed a highly potent enzymatic activity associated with preparations containing the virus of erythromyeloblastic leucosis in the dephosphorylation of adenosine triphosphate. Consideration of the problem is concerned not with the magnitude of the reaction but with the question of the identity of the enzymatic component. The fact that nothing in normal chicken plasma is effective reveals a specific association of the enzyme with the erythromyeloblastic disease. Several possible origins of such an enzyme seem obvious: the material might be elaborated into the circulation by the tissues of the host, by the affected bone marrow or the specific primitive cells or other tissues not directly affected by the virus; cell fragments remaining in the plasma after filtration might act as units or contain incorporated enzymes; or the reaction might be specific to the virus itself.

It is notable that the constituent of plasma carrying the activity must be associated with particles of the size of the virus since the enzymatic effects are carried through those processes designed to select the virus. This would rule out materials of small molecular size that might be freed by tissues into the circulation. It is recognized that the plasma contains amorphous masses larger than the virus which pass through the filter and appear in the concentrates of the virus as masses or aggregates up to about 7 μ in diameter. In the purification procedures such masses are easily eliminated by brief ultracentrifugation in fields of 10,000 X g. It is evident, then, that the enzymatic activity is associated, in all of the experiments thus far made, with particles essentially identical in sedimentation characters with the particles which are regarded as the virus. This is clearly shown in Fig. 1 in which the partition of enzymatic activity is parallel, within the limits of biological assay, with the distribution of virus infectivity among the ultracentrifugal fractions, as shown in titration experiments which will be described in another report. It is not inconceivable that the virus preparations contain a particulate component the size of the virus derived through possible breakdown of the primitive cells in the circulation. At this point, it is well to recall the experiments of Kielley and Meyerhof(7) who isolated from the muscle of the rat a particulate material with which there was associated the capacity to cause dephosphorylation of adenosine triphosphate. The sedimentation and other properties of this material seem, at least superficially, similar to analogous properties of the virus concentrates. While the possible contribution of such particles cannot be ruled out entirely at the moment, it can be said that in every respect in which the purified particulate material specific to erythromyeloblastic leucosis can be interpreted as the virus so can these same particles be regarded as the source of the enzyme freeing phosphorus from adenosine triphosphate.

The implications of these results, if fully established in further work, are of much significance. Evidence of enzymatic and metabolic activity of viruses in the absence of cells is so limited that this lack in constitution is regarded, in general, as a deficiency characteristic of this group of infectious agents. It is notable in this respect that while vaccinia elementary bodies give no evidence of such metabolic activity(8), the virus shows the presence of copper(9), flavin(10) and biotin (11), suggesting the existence of a limited respiratory system. Other enzymes found (12,13) in association with vaccinia virus have been viewed(13) as possible adsorbed contaminants carried through the purification procedures. Intensive investigation has shown that the influenza virus possesses the capacity to alter the physical structure(14) of the egg white inhibitor of hemagglutination and to liberate constituents(15,16) of this and other inhibitors of the reaction. It is noteworthy that this activity of influenza virus is likely to be of limited significance in comparison with that of enzymatic constituents which may be involved in physiological and reproductive processes of viruses. The capacity of the virus of erythromyeloblastic leucosis to react with adenosine triphosphate *in vitro* suggests possession by the virus of the immediate means for providing the energy, not only for utilization of intracellular materials but for making its entrance into the cell. It scarcely needs saying that the present experiments are open to criticism that the enzyme may be adsorbed on the virus particles. Such possible adsorption assumes considerable significance in itself, however, for it is evident that it must have occurred within the host cell since none of the enzyme of small molecular dimensions was present in the plasma. These possibilities make necessary further investigation, which is already underway.

Summary. A highly potent capacity for the dephosphorylation of adenosine triphosphate has been observed with preparations containing the virus of avian erythromyeloblastic leucosis. This enzymatic reaction occurred with the filtered plasma of birds diseased with the virus and with virus concentrates obtained from the plasma by ultracentrifugal procedures. The partition of enzymatic activity in these preparations has closely paralleled the distribution of virus infectivity of analogous materials measured in other work by titration of the virus in susceptible host chicks. No evidence of the reaction was obtained with the plasma from normal chickens. These experiments demonstrate the specific relationship of the dephosphorylation of adenosine triphosphate with avian erythromyeloblastic leucosis and indicate that the activity is a property of the etiological virus.

The authors are indebted to Dr. W. F. H. M. Mommaerts for his advice and helpful suggestions in the course of this work.

---

1. Jungherr, E., Doyle, L. P., and Johnson, E. P., *Am. J. Vet. Res.*, 1941, v2, 116.

2. Beard, D., Eckert, E. A., Csaky, T. Z., Sharp, D. G., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1950, v75, 533.

3. Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W., *J. Bact.*, 1952, v63, 151.

4. Mommaerts, W. F. H. M., in McElroy, W. D., and Glass, B., *Phosphorus Metabolism,* Vol. 1, The Johns Hopkins Press, Baltimore, 1951.

5. Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Cancer Inst.*, 1951, v12, 447.

6. Berenblum, I., and Chain, E., *Biochem. J.*, 1938, v32, 295.

7. Kielley, W. W., and Meyerhof, O., *J. Biol. Chem.*, 1948, v176, 591.

8. Parker, R. F., and Smythe, C. V., *J. Exp. Med.*, 1937, v65, 109.

9. Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1941, v74, 69.

10. Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1941, v74, 133.

11. Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., PROC. SOC. EXP. BIOL. AND MED., 1940, v45, 669.

12. Macfarlane, M. G., and Salaman, M. H., *Brit. J. Exp. Path.*, 1938, v19, 184.

13. Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1942, v76, 163.

14. Eckert, E. A., Lanni, F., Beard, D., and Beard, J. W., *Science*, 1949, v109, 463.

15. Gottschalk, A., and Lind, P. E., *Nature*, London, 1949, v164, 232.

16. Burnet, F. M., *Physiol. Rev.*, 1951, v31, 131.

Received February 19, 1952. P.S.E.B.M., 1952, v79.

Are Foamy Cells in Atheromata of Reticulo-endothelial Origin? (19410)

PAUL N. HARRIS.

From the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

Although plausible reasons have been advanced for believing that cholesterol esters are carried into the atherosclerotic vessel wall by macrophages(1-3), more recent studies support another view. Kuntz and Sulkin(4) concluded that foam cells of atheromata arose *in situ* from vascular endothelium, and in rabbits with early atherosclerosis given India ink or lithium carmine intravenously thrice at 2-day intervals they observed pigment granules in foam cells in the liver and spleen, but not in atheromata. The interval between the last injection and death of the animals was not stated, but presumably was short. Duff and McMillan(5) gave Thorotrast to atherosclerotic rabbits at intervals of 5 minutes to 4 days before death. Thorotrast was found in foam cells in the superficial part of the plaques and in some endothelial cells overlying the plaques. Simonton and Gofman(6) administered Thorotrast to rabbits, and subsequently began cholesterol feeding. Thorium-containing phagocytes in the liver and spleen engulfed India ink injected 24 hours before death. Radioactivity in atheromata was low, and the authors concluded that macrophage migration was not a significant source of foam cells of atheromata.

In view of the brevity of the interval between labeling of the reticulo-endothelial cells and death in the experiments cited, and since thorium-laden cells may perhaps migrate less readily than others, our experiments may be of interest.

Methods. Atherosclerosis was induced by incorporation in the diet of young adult rabbits of 3% of a 10% suspension of cholesterol in cottonseed oil. After 2 weeks, weekly intravenous injection of 3 cc of 17% Higgins India ink in normal NaCl solution was instituted. The duration of cholesterol administration was as follows: one rabbit, 3 months; 4 rabbits, 6 months; and one rabbit, 10 months. Two control rabbits were given ink for 10 months.

Results and discussion. There was a small intimal plaque in the ascending aorta of each control rabbit. The aorta of the rabbit that received cholesterol for 3 months was normal. The remaining animals had severe atherosclerosis. The reticulo-endothelial cells of all rabbits were laden with carbon. Although cells with foamy cytoplasm were abundant in the atheromata, no carbon-containing phagocytes were present. Absence of pigmented phagocytes from the aorta of the controls should be expected, since Leary(1) stated that macrophages in man and in experimental animals in which a great variety of forms of particulate matter have been engulfed exhibit no tendency to invade arterial walls.

While few animals were used, it was obvious that use of larger numbers would be futile. The results are more in accord with the view that cholesterol esters enter blood vessel walls by diffusion than with that that they are transported therein within migrating reticulo-endothelial cells.

Summary. Atherosclerosis was induced in rabbits by administration of cholesterol while the reticulo-endothelial cells were being laden

with carbon. Carbon-laden phagocytes were not found in the atheromata.

I am indebted to Messrs. John Owen and Henry West for technical assistance.

---

1. Leary, T., *Arch. Path.*, 1941, v32, 507.
2. Dauber, D. V., and Katz, L. N., *Arch. Path.*, 1943, v36, 473.
3. Gordon, I., *Arch. Path.*, 1947, v44, 247.
4. Kuntz, A., and Sulkin, N. M., *Arch. Path.*, 1949, v47, 248.
5. Duff, G. L., and McMillan, G. C., *Circulation*, 1950, v2, 465.
6. Simonton, J. H., and Gofman, J. W., *Circulation*, 1951, v4, 557.

Received December 26, 1951. P.S.E.B.M., 1952, v79.

Comparative Blood Volume Changes in Anti-Platelet Serum Shock and Other Types of Shock.* (19411)

W. O. CRUZ, H. MOUSSATCHÉ, AND H. P. OLIVEIRA.

From the Departments of Haematology and Physiology, Instituto Oswaldo Cruz, Rio de Janeiro, Brasil.

Haematological changes observed(1) in anti-platelet serum shock, have indicated that haemodilution occurred in this type of shock. It seemed desirable, therefore, to study changes in the absolute volumes of red cells and plasma, respectively. Studies are presented in this paper which show results in anti-platelet serum, histamine, trypsin and peptone shock.

Methods. All experiments were done with dogs. Plasma volumes were determined by the injection of Evans blue dye (T-1824). Since radioactive substances were not available, another method was employed(2) for the determination of total cell volume, using red blood cells with methaemoglobin. The amount of anti-platelet serum injected for inducing shock varied with the titer of the serum.

Results. Table I shows plasma, red blood cell, and total blood volume determinations in different types of shock. In all observations the total blood volume diminished in anti-platelet serum shock. Identical results were observed in histamine shock. These results are in agreement with those of other authors who have studied histamine and other types of shock. Increase of total blood volume was observed in 2 dogs shocked by trypsin; the significance of these data requires further study.

Plasma volume diminished in all the animals, except those shocked by trypsin, in which it increased. The total red cell volume increased in both animals with tryptic shock, in one case of anti-platelet serum shock and in one of histamine shock. In all other observations (11 out of 16) the total red cell volume diminished (Table II). The ratio between the decreases of plasma and red cell volumes varied according to the type of shock. In anti-platelet serum shock the red cell volume decreased more than the plasma volume. This occurred occasionally in histamine shock also, but generally the reverse was observed, *i.e.*, a more accentuated decrease of the plasma than the red cell volume.

When methaemoglobin-labeled red cells were injected after shock had been induced, a greater proportion of methaemoglobin remained in the circulation than when a comparable amount of labeled cells were injected in normal animals (Table III). Since the number of labeled cells given before and after shock was not identical, a correction was used assuming that there was a linear relationship between the amount of methaemoglobin administered and that found in the peripheral blood. That such an assumption is valid is shown by the following experiment performed 3 times on the same dog:

Day of exp.	MetHb inj.	Circulating MetHb/100 g Hb
1	1.5	3.6
2	2.5	5.2
3	5.2	10.3

---

* Aided by a grant from Dr. Guilherme Guinle.

TABLE I. Absolute Values of Blood Volume Determinations Before and After Different Types of Shock in 16 Dogs.

Dog No.	Body wt, kg	Type of shock	Dose/kg body wt	Normal: Haemoglobin, g	Normal: Haematocrit, %	Normal: Blood vol (ml) Plasma	Normal: Blood vol (ml) Cells	Normal: Blood vol (ml) Total	Normal: Body haematocrit, %	Shock: Haemoglobin, g	Shock: Haematocrit, %	Shock: Blood vol (ml) Plasma	Shock: Blood vol (ml) Cells	Shock: Blood vol (ml) Total	Shock: Body haematocrit, %
1	9	A.P.S.	.3 ml	8.4	33	563	253	816	31	—	—	493	178	671	27
2	6.3		.6	12.4	44	292	191	483	40	—	—	283	72	355	20
3	8.2		.6	13.4	42	301	321	622	52	—	—	290	101	391	26
4	6.8		.8	13.8	45	328	451	779	58	8.3	27	271	170	441	39
5*	7		.4	11	38	471	338	809	42	12.7	44	320	367	687	53
6	9.3		1	9.8	34	434	172	606	28	8.4	29	378	100	478	21
7	10.1	H	2 mg	7.2	21	806	133	939	14	8.9	26	526	150	676	22
8	9.5		2	6.8	27	581	165	746	22	8.3	33	242	142	384	37
9	8.3		1.5	10.8	35	416	285	701	41	12.7	41	373	126	499	25
10	7.7		1.5	6.2	26	409	151	560	27	8.1	34	324	115	439	26
11	7.7		1.5	6.8	24	396	235	631	37	9.1	32	294	105	399	26
12	6.7		2	11	41	330	187	517	36	9.4	35	186	187	373	50
13	12.8		2	12.8	40	531	416	947	44	16.6	52	297	257	554	46
14*	8.3	T	5	11.2	37	333	156	489	31	13.9	46	384	200	584	34
15	5.5		5	11.4	42	250	259	509	51	13.8	51	290	342	632	54
16	9.1	P	300	11.2	41	471	330	801	41		—	312	318	630	51

* Since in these animals the blood pressure, 10 min after inj., returned to a level above 50 Hg mm, they were not computed in the analysis of results.
A.P.S. = Anti-platelet serum, H = Histamine, T = Trypsin, P = Peptone.

TABLE II. Percent Differences of Blood Volume Determinations Before and After Different Types of Shock in 16 Dogs.

Dog No.	Type of shock	Blood volume: Plasma	Blood volume: Cells	Blood volume: Total	Total body haematocrit (%)
1	A.P.S.	—12	—30	—18	—13
2		— 3	—62	—26	—50
3		— 4	—69	—37	—50
4		—17	—62	—43	—33
5*		*—32	+ 8	—15	+26
6		—13	—42	—21	—25
7	H	—35	+12	—28	+57
8		—76	—14	—49	+68
9		—10	—56	—29	—39
10		—21	—24	—22	— 4
11		—26	—55	—37	—30
12		—44	0	—28	+39
13		—44	—38	—41	+ 5
14*	T	*+15	+13	+72	+10
15		+16	+32	+24	+ 6
16	P	—34	— 4	—21	+24

* See Table I.

TABLE III. Circulating Methaemoglobin % (Ratio $\frac{MHb \times 100}{Hb}$) Before and After Different Types of Shock, Supposing the Same Amount of MHb Were Injected in Both Instances.

Dog No.	Type of shock	Avg MHb used before and after shock (g)	Methaemoglobin (%) Before shock	Methaemoglobin (%) After shock	% diff.
1	A.P.S.	3.4	5.4	6.3	+ 17
2		2.3	4.3	10.7	+168
3		2.3	2.8	7.5	+166
4		2.8	2	5.3	+165
5*		5	4.7	5.1	+ 6
6		2.6	5.5	9.5	+ 73
7	H	2.9	6.4	7	+ 9
8		3.7	9	9.1	+ 1
9		3.3	3.9	9.4	+ 49
10		3	8.2	11.2	+ 37
11		2.5	3.8	8.5	+124
12		3.6	7.4	7.4	0
13		3.2	2.4	3.9	+ 63
14	T	2	4.2	3.5	— 17
15		2.4	3.4	2.5	— 26
16	P	3	2.6	2.7	+ 4

* See Table I.

It is noteworthy that in anti-platelet serum shock, the proportion of circulating methaemoglobin was greater than that observed in other types of shock. A lesser proportion of circulating methaemoglobin was found in the 2 animals with tryptic shock.

Discussion. It is evident that the blood volume decreased in anti-platelet serum shock

(Table I) as well as in histamine and peptone shock as shown by others. Decrease in blood volume is one of the most common features in shock and has been found by many workers in several types of shock. There is no agreement on the mechanism of the decrease; one of the most widely accepted theories suggests leakage through the capillaries with loss of plasma. More recently, however, this theory has been criticized on the basis of the work of Fine and collaborators(3), who while studying shock induced by hemorrhage, burn and trauma observed stagnation of plasma within the capillaries in the peripheral blood. These authors found that after the injection, during deep shock, of a known amount of plasma tagged with radioactive substances (S, I, Br), the plasma volume determination was always less than that expected from the plasma volume before shock plus the amount of plasma injected. After previous washing with saline to remove the tagged plasma from the vessels, these tissues showed no radioactivity, indicating that no leakage of plasma occurred under the conditions studied. They described the situation picturesquely in these words: "If in the late phase of shock the peripheral blood is not in active circulation, in the same sense as the peripheral waters of a swamp through whose center a brook runs, are not in active circulation, complete mixing may not occur in the time interval allowed." Regarding red blood cells, Gibson, Seligman, Evans and Fine (4) found that after the injection of red blood cells tagged with radioactive iron administered into animals in deep shock, the untagged and tagged red cell content of the tissues indicated that one fifth of the capillary blood becomes stagnant or trapped out of the active circulation.

Our results, excepting in tryptic shock, indicated a decrease of both plasma and red cell volumes, the absolute changes varying according to the type of shock; in anti-platelet serum shock the red cell volume decreased more than the plasma volume, leading to an apparent striking hemodilution; on the other hand, in histamine and peptone shock the plasma volume decreased more than the red cell volume, leading to the classical hemoconcentration described by others. The two exceptions observed in the dogs shocked by histamine, where hemodilution was found, could perhaps be explained by the anemic condition of the animal, since it is known that the ratio between capillary and total body hematocrit is even greater in anemic than in normal dogs(5). Our observations in 2 dogs shocked by trypsin seem to indicate a condition peculiar to this type of shock, in which an unexpected increase of plasma and red cell volumes was found. These observations should be confirmed.

Since hemolysis or hemorrhage did not occur during the phase of shock observed and the decrease of red blood cells of samples taken from great vessels occurs one or two minutes after the administration of anti-platelet serum(1), trapping seems to be a justifiable explanation of such rapid blood changes. The evidence presented by Fine and collaborators(4) for trapping of red blood cells in the peripheral bed, and of Chambers and Zweifach(6) for trapping of plasma, offers a new explanation of the mechanism of the changes in the relationship between red cell and plasma volumes.

The results obtained by the injection of methemoglobin labeled red cells before and after the induction of shock (Table III) support the possibility of red blood cell trapping. A relative increase of the circulating methemoglobin, greater than would be expected, was observed in all our experiments excepting in tryptic shock.

These results make it clear that the determination of the jugular hematocrit is far less significant than is usually accepted. Even the total body hematocrit is not enough to describe the whole picture of the blood changes. For instance, dogs 13 and 15, respectively shocked by trypsin and histamine, both presented practically the same blood concentration (+6% and +5%), notwithstanding the decrease of the red blood cell and plasma volumes in the animal shocked by histamine and the increase in red blood cell and plasma volumes in the animal shocked by trypsin. The different conditions in these two animals is clearly shown in Table III. The increase of circulating red cells and plasma in dog 15 is evident in the result of —26% below the expected methemoglobin figure, while dog 13 shows plasma trapping in the result of +63% above the expected methemoglobin value. Thus, it is incorrect to accept the occurrence of hemodilution or hemoconcentration from determination of the hematocrit of blood in the great vessels. On the contrary, the results of these experiments indicated that different concentration of red cells can occur in the vessels; hemoconcentration in the great vessels with hemodilution in some of the capillaries and vice versa. Thus to explain the apparent hemoconcentration observed in the great vessels it is not necessary to assume extravasation of plasma or water nor to explain hemodilution is it necessary to assume loss of red cells from the circulatory system. To explain hemodilution in the great vessels by trapping, presupposes the stagnation of hemoconcentrated blood in the capillaries of some regions of the body and vice versa for hemoconcentration.

Summary. Total red blood cell volume (using red cells tagged with methemoglobin as a tracer) and plasma volume (using Evans blue dye T-1824) of dogs in different types of shock have been determined. Decreases in total red cell and plasma volumes have been observed in anti-platelet serum shock. The decrease in the red cell volume was greater than of the plasma volume, while in histamine shock the reverse was observed. Assumption has been made that trapping of red cells explains the apparent hemodilution in the great blood vessels in anti-platelet serum shock. The degree of trapping of the red cells, was determined by the proportion of circulating methemoglobin before and after shock. Analysis of the results shows the greater importance of the consideration of absolute values of red cell and plasma volumes rather than the acceptance of results indicated by the hematocrit of the blood from the great vessels, for the interpretation of the pathogenesis of shock.

1. Cruz, W. O., Moussatché, H., *Acta Haematologica,* 1951, v6, 56.

2. Cruz, W. O., Oliveira, H. P., in press.

3. Fine, J., Seligman, A. M., and Frank, H. A., *Surgery,* 1943, v14, 238.

4. Gibson, J. G., Seligman, A. M., Peacock, W., Fine, J., Aub, J., and Evans, R., *J. Clin. Invest.,* 1947, v26, 126.

5. Cruz, W. O., Silva E. M., and Pimenta de Mello, R., *Rev. Brasil. Biol.,* 1945, v5, 139.

6. Chambers, R., Zweifach, B. W., and Lowenstein, B. E., *Ann. Surg.,* 1944, v120, 791.

Received January 10, 1952. P.S.E.B.M., 1952, v79.

Experimental Porphyria. I. Isolation of Uroporphyrin I from Bone Marrow of Lead Poisoned Rabbits.[*] (19412)

RUDI SCHMID,[†] BARBARA HANSON, AND SAMUEL SCHWARTZ.
(Introduced by C. J. Watson.)

From the Department of Medicine, University of Minnesota Hospitals, Minneapolis, Minn.

It is generally believed that lead interferes with hemoglobin synthesis. Associated abnormalities in porphyrin metabolism, namely, the presence of increased erythrocyte protoporphyrin and of urinary coproporphyrin III have been repeatedly documented. More recently, studies in this laboratory have revealed the occurrence of marked increases in the concentration of coproporphyrin III in the bone marrow of lead-poisoned rabbits(1). In the course of these studies, large amounts of an ether-insoluble, uro-type porphyrin were found in the marrow of the same animals. The present report is concerned with the iso-

---

[*] This work was supported in part by the Atomic Energy Commission, Contract Nos. AT(11-1)-100 and AT(11-1)-108.

[†] Clinical Fellow of the American Cancer Society.

lation and identification of this latter porphyrin.

Experimental. The effect of lead on bone marrow and blood porphyrin concentrations has been studied in 23 rabbits. Five of these (No. 60, 93, 94, 95, and 96) were given single subcutaneous injections of 100 mg of lead acetate (63.7 mg of lead) per kg of body weight, and were killed for tissue porphyrin analyses 25 to 44 days later. Rabbits No. 62, 64, 65, 77, and 88 were injected subcutaneously with 100 mg of lead acetate per kg 25 days prior to sacrifice and examination. For this period of 25 days, each rabbit received daily intravenous injections of an aqueous solution of 5 mg of crystalline riboflavin phosphate.‡ Rabbits No. 62 and 88 were also given daily intravenous injections of 500 mg of ascorbic acid. Two animals (No. 91 and 99) were repeatedly given small amounts of lead acetate intravenously over periods of several weeks prior to the examination. The total of the injected lead acetate amounted to 85 and 100 mg, respectively. Eleven rabbits were given repeated subcutaneous injections of phenylhydrazine (20-40 mg per injection) over periods of 6 to 45 days. Total dosage ranged from 110-400 mg. On the day of the last phenylhydrazine administration they were injected intravenously with 20 mg of lead acetate per kg of body weight. Four animals (No. 105, 106, 210, and 214) were sacrificed 3 days later for blood and bone marrow porphyrin studies. Seven (No. 212, 213, 216, 219, 220, 221, and 222) were sacrificed for study after 7 to 9 days. After bleeding the animals the bone marrow of the long bones was recovered and the copro- and protoporphyrin extracted according to the method previously described (1). The ethyl acetate and glacial acetic acid extract was washed 3 times with 3% sodium acetate. In all instances the sodium acetate solution contained an ether insoluble uro-type porphyrin which was also insoluble in ethyl acetate and butyl alcohol at a pH of approximately 6. At a pH of approximately 4 (gray to congo paper), however, the uroporphyrin was readily extracted from the aqueous solution by either of the latter two solvents. This difference in solubility was taken advantage of in further purification. The 3% sodium acetate extract containing the uro-type porphyrin was acidified with acetic and hydrochloric acids to a pH of approximately 4, and the porphyrin was extracted by ethyl acetate. For fluorimetry, the porphyrin was reextracted with 1.5 normal hydrochloric acid.

Results. The important data are summarized in Tables I and II in terms of porphyrin concentrations in μg per 100 ml of packed cells. No correction factor has been applied for the non-erythropoietic cells contained in the marrow. In several of the animals the liver and kidneys were also examined for porphyrins. Since the amounts of uro- and coproporphyrin found in these organs were insignificant compared with the values for the bone marrow, they are not included in these tables. Fluoromicroscopic examination of liver sections revealed essentially no red fluorescence, whereas the immature erythropoietic cells of the bone marrow and the peripheral blood showed the typical bright red fluorescence of porphyrin(2-5). As seen in Tables I and II, marked elevation of uro-type porphyrins were observed in the bone marrow of all lead intoxicated rabbits. In the circulating red cells, however, this porphyrin was found in significant amounts only in those rabbits pretreated with phenylhydrazine and killed shortly after administration of the lead. The administration of riboflavin with or without ascorbic acid had no significant effect on the results obtained. The values obtained for erythrocyte and bone marrow coproporphyrin and protoporphyrin are essentially similar to those previously reported for lead poisoned rabbits(1). It is seen that elevation of erythrocyte coproporphyrin occurred only in those rabbits with acute lead poisoning.

As seen in Table I, the repeated administration of lead acetate intravenously resulted in porphyrin concentrations similar to those observed in the rabbits given only a single subcutaneous injection of lead acetate.

Isolation of uroporphyrin I from the bone marrow. The bone marrow uroporphyrin has

---

‡ Provided through the courtesy of Hoffmann-LaRoche Co.

TABLE I. Erythrocyte and Bone Marrow Porphyrins in Rabbits with Chronic Lead Poisoning.

Rabbit No.	Erythrocytes			Bone marrow			Remarks
	Uro-	Copro-porphyrin	Proto-	Uro-	Copro-porphyrin	Proto-	
			In μg per 100 ml cells				
60	0	5.9	233	437	522	125	Subcutaneous lead acetate
94	0	.7	137	15	194	143	
93	0	1.2	157	28	246	105	
96	0	13.8	348	147	490	337	
95	0	2	165	69	121	100	
64	0	4.8	269	678	781	325	Riboflavin
65	0	3.4	168	423	334	105	
77	0	4.8	235	160	319	135	
88	0	34.6	237	375	392	128	Riboflavin and ascorbic acid
62	0	8.1	227	435	510	143	
91	0	2	404	75	248	176	Intrav. lead acetate
99	0	4.4	525	74	298	164	
*	0	.5 (.4-.6)	83.3 (58-106)	?tr	4.5 (1.7-8.6)	87.5 (54-137)	Normal control

* Mean values for 3 normal control rabbits (No. 44, 51, 57).

TABLE II. Erythrocyte and Bone Marrow Porphyrins in Rabbits with Acute Lead and Phenylhydrazine Poisoning.

Rabbit No.	Erythrocytes			Bone marrow			No. days animals killed after lead acetate inj.
	Uro-	Copro-porphyrin	Proto-	Uro-	Copro-porphyrin	Proto-	
			In μg per 100 ml cells				
105	44	274	1410	118	971	371	3
106	31	488	942	141	1410	299	
210	149	587	1135	117	1056	252	
214	292	594	450	844	1018	339	
212	6.4	26.2	785	376	1782	328	7-9
213	1.7	9.8	527	55	440	247	
216	0	1.4	376	79	396	355	
219	3.4	3.4	198	443	903	960	
220	3.9	4.1	411	482	1303	409	
221	1.5	4.4	360	139	594	138	
222	70.3	321	1153	114	416	521	
*	0	60.5±41.5	137±19	tr—4.1	68.5±25.5	110±44	Phenylhydrazine control

* Mean values for 2 control rabbits with phenylhydrazine anemia alone (No. 145 and 207).

been crystallized on two occasions. All available bone marrow from 3 rabbits with lead intoxication (No. 39, 71, and 74) was pooled and the uroporphyrin extracted and purified as previously described. It was esterified by mixing the final concentrate with approximately 20 volumes of a solution of methyl alcohol-concentrated sulfuric acid (19:1). The methyl ester was taken into chloroform and further purified by calcium carbonate chromatography as previously described(6). It was then crystallized from chloroform: methyl alcohol. The crystals had the characteristic hair-like form of uroporphyrin I methyl ester. The melting point was 278 to 282°C (corrected) with formation of droplets at the latter temperature. The Soret band absorption maximum in chloroform solution (4060 Å) was identical with that of purified uroporphyrin I methyl ester isolated from urine of a patient with congenital or erythropoietic porphyria. From this it is evident that at least as far as the formation of this porphyrin is concerned, the present findings deserve the designation of "experimental porphyria."

In a second experiment 8 rabbits with experimental lead intoxication were given 90 mg of phenylhydrazine in 3 divided doses over a period of 8 days prior to the examination. A pool of 55 g of bone marrow was obtained. Isolation and estimation of the different porphyrins yielded the following values per 100 g of marrow: Uroporphyrin, 458 μg; coproporphyrin, 815 μg, and protoporphyrin, 300 μg.

Uroporphyrin I was again crystallized as the methyl ester. The melting point was 283 to 286°C (corrected). The absorption maximum in chloroform was again at 4,060 Å, and the fluorescence spectrum maximum as determined in a Steinheil analytical microspectroscope was identical with that of uroporphyrin I methyl ester.

The type III isomer was found to constitute 96% of the total coproporphyrin[§] isolated from the bone marrow of these rabbits. It was crystallized in form of its methyl ester from chloroform:methyl alcohol. The characteristic rosette-form crystals were easily soluble in cold ether.

Discussion. The presence of traces of uroporphyrin has been reported in human urine (7,8) and tissues in post fetal life(9). The occurrence of larger amounts has generally been considered pathognomonic of porphyria. The normal occurrence in certain species such as the fox squirrel (*Sciurus niger*)(10), the turacos(11,12), and molluscs(13) has been well documented. The reported occurrence of traces of uro-type porphyrins in normal human urine and in a variety of experimental conditions will be considered in a separate communication.

It is believed that this is the first report of the isolation of crystalline uroporphyrin under experimental conditions. The crystallization of the type I isomer of uroporphyrin from the bone marrow of lead-poisoned rabbits is especially surprising, since the coproporphyrin isolated from the marrow and the urine of these rabbits is nearly all the type III isomer. The exact mechanisms responsible for this accumulation of two different isomer types are unknown. Further studies relating the above findings to human porphyria are described in the companion paper.

Summary and conclusions. 1. Crystalline uroporphyrin I has been isolated from the bone marrow of lead poisoned rabbits and found to be identical with uroporphyrin I isolated from the urine of a patient with photosensitive (erythropoietic) porphyria. 2. The bone marrow of rabbits with acute or chronic lead poisoning has been found to contain from 15 to 844 μg of uroporphyrin I per 100 ml. 3. In experimental lead poisoning, significant amounts of uroporphyrin are found in the circulating red blood cells only during the very acute stage. Erythrocyte coproporphyrin is likewise increased markedly only during the acute phase.

[§] Determined by the fluorescence-quenching method (Schwartz, S. and co-workers, *J. Biol Chem.*, 1947, v168, 133).

1. Schmid, R., Schwartz, S., and Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 705.
2. Seggel, K. Ad., *Ergeb. inn. Med. Kinderheilk.*, 1940, v58, 582.
3. Borst, M., und Koenigsdoerffer, H., *Untersuchungen ueber Porphyrie, Hirzel*, 1929.
4. Mueller-Neff, H., *Folia Haematol.*, 1937, v56, 18.
5. Emminger, E., und Battistini, Z., *Virchow's Archiv.*, 1933, v290, 492.
6. Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, 1945, v157, 323.
7. Fischer, H. and Duesberg, R., *Arch. exp. Path. Pharmakol.*, 1932, v166, 95.
8. Nicholas, R. E. H., and Rimington, C., *Scand. J. Clin. and Lab. Invest.*, 1949, v1, 12.
9. Kluever, H., *Science*, 1944, v99, 482.
10. Turner, W. J., *J. Biol. Chem.*, 1937, v118, 519.
11. Fischer, H. and Hilger, J., *Z. physiol. Chem.*, 1924, v138, 49.
12. Rimington, C., *Proc. Roy. Soc. London*, 1939, v127B, 106.
13. Fischer, H., and Hofmann, H. J., *Z. physiol. Chem.*, 1937, v246, 15.

Received January 14, 1952. P.S.E.B.M., 1952, v79.

Experimental Porphyria. II. Type Produced by Lead, Phenylhydrazine and Light.* (19413)

S. SCHWARTZ, M. KEPRIOS, AND R. SCHMID.† (Introduced by C. J. Watson.)

From the Department of Medicine, University of Minnesota Hospital, Minneapolis

Glycine and acetate have been shown to be specific precursors of hemoglobin protoporphyrin(1), and glycine at least, is a known precursor of the uro- and coproporphyrins excreted by patients with porphyria(2). In order to study the biological interrelationships of these compounds, it would be most useful to have available an experimental animal which produced and excreted large amounts of porphyrin. Such an animal might also serve as a valuable source of radioactive porphyrins following administration of C^{14} labelled glycine or acetate. Turner has shown(3) that the fox squirrel (*Sciurus niger*), has a physiological porphyria. Preliminary studies with several of these animals indicated, however, that the practical difficulties involved in handling them limited their usefulness. Major attention was therefore directed at stimulating porphyrinuria in the rabbit, which has been shown to respond well to a variety of agents. Thus, for example, the repeated administration of phenylhydrazine to rabbits increases their urinary coproporphyrin excretion to nearly 100 μg per day from control levels of approximately 30 μg per day. Lead produces the most marked and most prolonged coproporphyrinuria of any agent known(4), especially if the rabbit is exposed to sunlight or to light from a mercury lamp(5). Peak values of up to 800 μg per day have been observed following this treatment. Finally, Pimenta de Mello has recently shown that a photodynamic dye, Rose Bengal, given in conjunction with ultraviolet light increases the urinary coproporphyrin excretion to approximately 100-300 μg per day(6). As regards previous attempts to induce porphyria in the rabbit it might be noted that sulfonal has been reported to produce an inconstant excretion of ether-insoluble porphyrin (? uroporphyrin)(7,8). No quantitative data, however, are available, and this porphyrin has not been crystallized. The isolation of uroporphyrin I from the bone marrow of rabbits treated with lead alone or with phenylhydrazine plus lead is described in a companion paper(9). Although the present studies were undertaken both to extend these tissue studies and to stimulate the urinary excretion of porphyrins to as marked a degree as possible, their most interesting result has been the production in rabbits of a temporary condition which has chemical features characteristic of human porphyria. It is the purpose of the present communication to describe the features of this experimental "porphyria" of rabbits.

Methods. The recently described ("5 ml") method(10) was employed for the determination of urinary coproporphyrin. Since rabbit urine is normally alkaline, no sodium carbonate preservative was used. Values obtained by this method averaged about twice those observed in duplicate samples which employed our previously used ("long") method. This is similar to our experience with human urine(10). Urinary uroporphyrin was determined in 5-10 ml samples of urine as follows: The urine was barely acidified with glacial acetic acid and heated in a boiling water bath for 30 minutes to convert uroporphyrin precursors. Five ml of buffered acetic acid (4 volumes glacial acetic acid plus 1 volume of saturated aqueous sodium acetate) and 30 ml of water were added and the solution shaken well with 100 ml of ethyl acetate. The ethyl acetate was washed once with 30 ml of 0.005% aqueous iodine solution and 3 to 6 times with 30 ml portions of 3% sodium acetate (or until no further red fluorescence was extracted by the wash solution). The ethyl acetate, containing the coproporphyrin,

---

* Aided by grants from the Atomic Energy Commission, Contract Nos. AT(11-1)-108, and AT (11-1) -100, and from the Graduate School, University of Minnesota. Presented in part before the Central Society for Clinical Research, Nov. 2, 1951.

† Clinical Fellow, American Cancer Society.

was discarded,[‡] and the washes were added to the initially extracted urine for further study of the uroporphyrin. Most of the data reported here employed the Sveinsson method (11) for further purification and concentration of the uroporphyrin for fluorimetric analysis. Because variable and often considerable losses were encountered with this method another procedure has been employed more recently. The combined aqueous solution was filtered through an approximately 5 cm high and 2 cm wide column of aluminum oxide (Merck: for chromatography) to adsorb the uroporphyrin. The aluminum oxide was next washed with half-saturated sodium acetate solution until essentially no further blue-green fluorescence was removed as determined by a blank reading by fluorimetry. After further washing with about 20 ml of water, the uroporphyrin was eluted with 1.5 N HCl, and its concentration determined fluorimetrically(10). Because the uroporphyrin standards are relatively unstable, it is suggested that fluorimetric analyses be made by comparison with a standard solution of coproporphyrin which has been calibrated with freshly prepared solutions of uroporphyrin. Data on the accuracy and limitations of this procedure will be described in a separate communication. It might be noted here, however, that there is considerable error at low levels of uroporphyrin concentration as found in normal rabbit urine because of nonspecific blue-white fluorescence. At higher concentration levels (*i.e.,* above approximately 50 μg per day) a distinct red fluorescence is visible in the final hydrochloric acid eluate. The coproporphyrin isomer ratios were determined by the previously described fluorescence-quenching method(12). The presence of urinary porphobilinogen was ascertained by the chloroform insolubility of the Ehrlich-aldehyde compound(13). The further separation of the porphobilinogen into two components is described in the experimental section. The tissue copro- and protoporphyrins have been determined by the method previously described(14). The uroporphyrin present in the initial 3% sodium acetate washes was analyzed by the methods described above for the urinary uroporphyrin.

Experimental. A total of 69 rabbits have been treated with various combinations of phenylhydrazine, lead, Rose Bengal, and light. Twelve died within 24 hours after receiving lead intravenously. The following studies were made in the remaining rabbits: urinary porphyrins in 46, erythrocyte porphyrins in 27, marrow porphyrins in 23, and liver porphyrins in 10. Qualitative porphobilinogen reactions were studied in approximately 1/3 of the animals.

1. *Urinary porphyrins.* Detailed studies will be described for Rabbit No. 1 to illustrate the treatment and results obtained. These results are at least qualitatively typical of those observed in all rabbits given lead and other agents noted above. Rabbit No. 1 was injected with 20-40 mg of phenylhydrazine 8 times over a period of one month. He was then injected intravenously with 20 mg of lead acetate per kg of body weight, his back was shaved, and he was at once exposed for 30 minutes to unfiltered light from a CH-4 mercury arc lamp. Following a one-day period of anuria his 24-hour urinary coproporphyrin excretion rose to 1823 μg. Isomer analysis of this porphyrin showed it to be es-

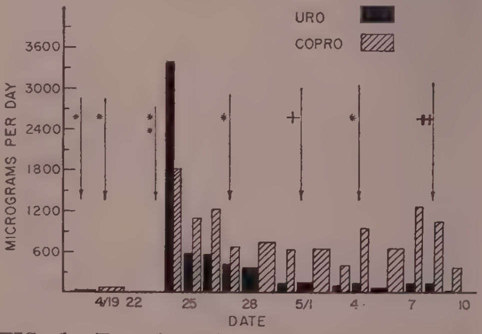


FIG. 1. Excretion of coproporphyrin and uroporphyrin in a rabbit treated with phenylhydrazine, lead and light. * 40 mg phenylhydrazine-HCl, subcutaneously (7 doses, 20-40 mg each, injected prior to 4/19). ** 40 mg phenylhydrazine-HCl, 20 mg lead acetate/kg body wt, and ½ hr light exposure (CH-4 mercury lamp). + ½ hr light exposure. ++ 20 mg lead acetate and ½ hr light exposure.

[‡] Variable amounts of coproporphyrin were lost on boiling; hence a separate analysis of unboiled urine was employed for this porphyrin.

TABLE I. Peak Urinary Porphyrin Values in Different Groups of Experimentally Treated Rabbits.

Group	No. rabbits	Copro—μg/day		Uro—μg/day	
		Range	Mean	Range	Mean
1. Normals	24*	14–86	41	8–49	25
2. R.B.‡ + light	7	58–158	86	9–38	23
3. Phen.§	11	29–118	78	14–104	40
4. Phen. + light	3	64–144	96	35–64	45
5. Pb	6	301–773	465	—†	124
6. Phen. + Pb	4	227–952	532	150–503	281
7. Phen. + Pb + light	12	358–1823	726	36–3466	625
8. Phen. + Pb + R.B. + light	11	429–1041	755	50–2280	466

* Total of 39 individual analyses. Mean coproporphyrin value = 26 μg/day; mean uroporphyrin value = 17 μg/day. As noted in text, the latter values are undoubtedly higher than the true values.

† Combined analyses for uroporphyrin on only 2 rabbits.

‡ Rose Bengal, 50-100 mg/kg intrav.

§ Phenylhydrazine –HCl, 2-17 subcut. injections, 20-40 mg each.

sentially all type III isomer. However, the major portion of the urinary porphyrin, amounting to 3466 μg, was unlike coproporphyrin in being insoluble in ether or ethyl acetate. This porphyrin was isolated as the methyl ester following purification by calcium carbonate chromatography(12). The long, wavy, hairlike crystals melting at 280-282° (corrected) established its identity as uroporphyrin I, the same isomer which is present in the urine of patients with the erythropoietic (congenital or photosensitive) type of porphyria(15). This was confirmed by decarboxylation of the uroporphyrin and isolation of the resulting coproporphyrin I whose methyl ester had a melting point of 246-250° (corr.). Isomer analysis of the mother liquor indicated that it too contained only coproporphyrin I.

Daily analyses of the urinary copro- and uroporphyrin were made with the results shown in Fig. 1. It will be noted that the uroporphyrin values, after rising sharply, fell rapidly while those of coproporphyrin fell more slowly. Secondly, it is seen that the administration of more phenylhydrazine, light or lead had relatively little further effect.

Urinary porphyrin findings in the other rabbits are summarized in Table I in terms of the range of peak values and their means seen with each type of treatment. As shown in this table, markedly elevated values for both porphyrins have been observed only following the combined use of lead with the various other agents noted. Only moderate coproporphyrinuria and inconstant increases in uroporphyrin excretion were produced by phenylhydrazine, while administration of Rose Bengal plus light affected the excretion of only coproporphyrin. Because of the large spread of values within each group and because of the many variables including differences in age, sex, dose, and time of year, no attempt is made at this time to interpret too rigidly the differences between the various lead-treated groups.

It should be pointed out that chromatographic analysis of the methyl esters of both porphyrin fractions revealed the presence of very small amounts of other unidentified porphyrins. The analyses are therefore not entirely specific for copro- and uroporphyrins.

The large majority of the urinary coproporphyrin was excreted in the free form. This was established first by spectroscopic observation, and secondly by the fact that about 90% of this porphyrin was extracted readily from ethyl acetate by 0.3 N HCl. The remaining porphyrin was clearly a metal complex, with absorption maxima at 538 and 578 mμ. This was extracted by 1.5 N HCl. This behavior is characteristic of zinc-coproporphyrin. In several instances, the "native" coproporphyrin and its non-fluorescing precursor were determined separately(16) in urine freshly removed from the bladder post mortem. The two were generally present in approximately equal amounts.

On 2 occasions, rosette-like crystals characteristic of coproporphyrin III were isolated from pooled samples of urine from several rabbits in groups 6-8 inclusive (Table I). Isomer analysis showed 89-98% of the total porphyrin to be the type III isomer.

2. *Urinary porphobilinogen.* The presence of a strongly positive Ehrlich reaction was noted in the urine of the first rabbit and confirmed in all rabbits studied subsequently. The red aldehyde compound formed was identified as porphobilinogen because of its chloroform insolubility, absorption spectrum (maxima at about 528 and 566 mμ[§]), and other properties as noted below. The porphobilinogen from several of the rabbits was purified by aluminum oxide chromatography as follows:[¶] An equal volume of acetone was added to the combined extracted urine (unboiled) and the 3% sodium acetate washes of the ethyl acetate. This was filtered through a column of aluminum oxide (Merck: for chromatography) and the adsorbed porphobilinogen eluted with 3% acetic acid in water. When the eluate no longer gave a positive Ehrlich-aldehyde reaction, the column was washed with 35% acetic acid. A second Ehrlich-positive fraction was then obtained. The red aldehyde compound was not extracted by chloroform from either fraction. The second fraction, however, differed from the first in being completely extracted by N butyl alcohol. This difference is not related to the difference in acetic acid concentrations since addition of acetic acid to the former eluate, or dilution of the latter with water does not alter this property. A similar separation has been observed in urine from a patient with intermittent acute porphyria. The significance of this porphobilinogen fractionation is being investigated further. No attempt has been made to estimate the amount of these porphobilinogens quantitatively. It was apparent, however, that their concentration was very low in the urine of rabbits treated with lead alone. In these rabbits the presence of porphobilinogen could be established with certainty only after concentration and purification by aluminum oxide chromatography. The additional administration of the other agents described, however, resulted in definite porphobilinogen reactions which persisted for several weeks.

3. *Tissue porphyrins.* In addition to the urinary studies, we have been especially concerned with determination of porphyrin concentrations in bone marrow, circulating red cells, and liver, because of the finding that the bone marrow appears to be the chief source of porphyrin in porphyria erythropoietica (congenital porphyria) whereas the liver seems to be the main source of localization of porphyrin in the hepatic forms of this disease as seen in acute intermittent and mixed porphyria(15). The companion paper(9) has discussed the effect of lead and of phenylhydrazine plus lead on bone marrow and red cell porphyrins. The general finding of elevated values has been confirmed for these two types of treatment. Highest bone marrow values have been found in 2 rabbits given Rose Bengal and ultraviolet irradiation in addition to phenylhydrazine and lead; coproporphyrin values of 1793 and 2084 μg per 100 g were observed respectively, while the uroporphyrin concentration in one rabbit was over 1500 μg %.

There was considerable variation in the amount of porphyrin in the bone marrow and circulating erythrocytes in different rabbits

TABLE II. Erythrocyte Coproporphyrin Concentration (μg %) in Rabbits Treated with Phenylhydrazine, Lead, and Light (Autumn, 1951).*

	Rabbit No.						
Day	56	59†	61	63	64	65†	66†
1	5	5	7	—	—	10	—
2	11	—	13	6	10	—	9
3	24	76	26	—	—	7	—
4	21	11	20	11	13	—	4
5	—	—	—	—	—	3	—
6	14	11	6	6	4	—	—
9	4	2	3	—	—	—	—

* Avg value in 13 normal rabbits = 2.6 μg %.

† A single injection of 30-40 mg lead acetate IV on day 0 followed repeated subcut. inj. of phenylhydrazine. The other rabbits were exposed, in addition, to unfiltered light from a CH-4 mercury lamp. No more than trace amounts of ethyl acetate insoluble porphyrin was noted in any instance. Analyses were performed on red cells from 6-9 ml of whole blood.

[§] Because the absorption bands are relatively broad and somewhat unstable, these measurements are to be considered approximate only.

[¶] The authors are indebted to Mr. Page Edmondson for his assistance in these studies.

TABLE III. Erythrocyte Protoporphyrin Concentration (μg %) in Rabbits Treated with Phenylhydrazine, Lead, and Light (Autumn, 1951).*

Day	Rabbit No. 56	59	61	63	64	65	66
1	183	155	122	—	—	100	—
2	418	—	309	179	210	—	378
3	816	1237	631	—	—	340	—
4	912	686	590	364	355	—	444
5	—	—	—	—	—	387	—
6	537	559	366	455	327	—	—
9	533	343	321	—	—	—	—

* See Table II for protocol.
Avg value in 13 normal rabbits = 54 μg %.

treated similarly. In general, erythrocyte coproporphyrin values were much lower in the autumn of 1951 than in the spring. As noted previously(9), highest values were observed during the first 3-4 days after administration of lead, as shown in Tables II and III.

In this regard it is of interest to note that Sumegi and Putnoky(17) described increased porphyrinuria in lead-poisoned rabbits in the spring but not in the autumn, and Seggel(18) described a similar phenomenon for fluoroescytes in lead-poisoned rabbits. This possible seasonal relationship deserves further investigation.

Fluorescence microscopy study of fresh unstained smears of bone marrow revealed the presence of numerous red-fluorescing cells on exposure of the smear to the 405 mμ light from an AH-6 mercury lamp. These studies will be described in more detail elsewhere.

Two rabbits have been injected with glycine-2-C^{14} following treatment with phenylhydrazine, lead, Rose Bengal, and ultraviolet light. Results of these isotopic studies will be described elsewhere. Among the most interesting findings was the separation of 7 red-fluorescing zones on chromatography of the esterified bone marrow porphyrins. Here too, then, the analytical separation of the porphyrin into copro-, uro-, and protoporphyrins is not entirely specific.

Complete liver porphyrin analyses have been performed in but 10 instances. In no case have more than trace amounts of uroporphyrin been found. The coproporphyrin concentrations were generally normal, being elevated above 20 μg % in but one instance; a value of 268 μg % was observed in a rabbit treated with phenylhydrazine, lead, Rose Bengal, and ultraviolet light. This rabbit died during the night, 12-20 hours after treatment. All other analyses were made at least 2 days after administration of the lead.

Discussion. The excretion of uroporphyrin has generally been considered abnormal, due as Garrod termed it, to an "inborn error of metabolism"(19). Recently, however, Nicholas(20) and others have renewed Fischer's earlier claim(21) that traces of a uro-type porphyrin are present normally in human urine. We have confirmed this finding in urine from normal rabbits, rats, and dogs, but have not isolated the porphyrin in crystalline form.

As pointed out in the previous section, phenylhydrazine alone may increase the urinary excretion of uroporphyrin, while much greater increases are observed following treatment with lead and ultraviolet light. It is not unlikely, too, that methods now available will demonstrate increased amounts of uroporphyrin in a wide variety of clinical conditions. If the presence of uroporphyrin remains the decisive criterion in the diagnosis of porphyria, one might ask, "At what concentration level shall we place the dividing line in defining porphyria?" Actually, it would seem that this diagnosis is best based on the simultaneous presence of certain specific clinical *and* chemical features. Hence the tentative use of ditto marks around "porphyria" in the title of the report, though from a purely chemical point of view the characterization of this experimental condition as porphyria is not an unreasonable one. We have thus far made no clinical studies except to note that several of the rabbits have developed paresis or paralysis of the hind limbs. Whether this is related only to the plumbism rather than the "porphyria" is not clear.

It will be noted that these rabbits exhibit certain features of each of the three types of human porphyria. They resemble cases of erythropoietic ("congenital" or "photosensitive") porphyria in excreting uroporphyrin I, mainly in the free form rather than the zinc complex, and in showing high concentrations of porphyrin in the bone marrow with relatively little in the liver. On the other hand, they are like cases of "intermittent acute" and "mixed" forms of hepatic porphyria in exhibiting a positive porphobilinogen reaction (though the porphobilinogen reaction is sometimes negative in mixed porphyria) and in excreting coproporphyrin which is predominantly the type III isomer. Their urine also contains increased amounts of urobilinogen for several days after administration of lead. They thus show evidence of liver dysfunction which is also present characteristically in cases of "mixed" porphyria.

Like erythropoietic or congenital porphyria patients with splenomegaly and hemolytic anemia, the rabbits have a hemolytic anemia due to phenylhydrazine, with associated stimulation of erythropoiesis and porphyrin production. The inhibited hemoglobin synthesis produced by lead, when superimposed on this stimulated porphyrin production, seems to be the important factor in causing the observed outpouring of urinary porphyrins and porphobilinogen.

Summary and conclusions. Treatment of rabbits with phenylhydrazine, lead, and light has been found to produce a temporary condition which is chemically similar to porphyria in humans. The following features are similar to those observed in human porphyria erythropoietica. a. Uroporphyrin I is excreted in the urine. Values of up to 3466 μg per day have been observed. b. The major portion of the urinary porphyrin is excreted as the free form rather than as the zinc complex. c. Bone marrow uro- and coproporphyrin values were elevated to 1500-2000 μg % as compared to normal values of approximately 0 and 5-10 μg % respectively. d. Liver porphyrin concentrations are generally normal.

The following chemical features are characteristic of porphyria hepatica. a. The urinary porphobilinogen reaction is positive. This porphobilinogen has been fractionated by aluminum oxide chromatography into 2 components. The aldehyde of only one of them is extractable by N butyl alcohol. b. The increased excretion of urinary coproporphyrin is nearly all due to the type III isomer. Values ranging up to 1823 μg per day have been observed.

The authors are indebted to Dr. C. J. Watson for his helpful support and encouragement of these studies.

1. Shemin, D., and Wittenberg, J., *J. Biol. Chem.*, 1951, v192, 315.

2. a) Grinstein, M., Aldrich, R. A., Hawkinson, V., and Watson, C. J., *J. Biol. Chem.*, 1949, v179, 983; b) Neuberger, A., Muir, H. M., and Gray, C. H., *Nature*, 1950, v165, 948; c) London, I. M., West, R., Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, 1950, v184, 365; d) Grinstein, M., Aldrich, R. A., Hawkinson, V., Lowry, P., and Watson, C. J., *Blood, J. Hematol.*, 1951, v6, 699.

3. Turner, W. J., *J. Biol. Chem.*, 1937, v118, 519.

4. Schwartz, S., and Zagaria, R., U. S. Atomic Energy Commission, Oak Ridge, Tenn., Declassified Document MDDC-504; *Toxicology of Uranium*, A. Tannenbaum, ed., National Nuclear Energy Series, Manhattan Project Technical Section, pp. 290-326.

5. De Mello, R. Pimenta, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 823.

6. ———, PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 292.

7. Neubauer, O., *Arch. Exp. Path. u. Pharmakol.*, 1900, v43, 456.

8. Waldenström, J., and Wendt, S., *Z. Physiol. Chem.*, 1939, v259, 157.

9. Schmid, R., Hanson, B., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, ——.

10. Schwartz, S., Zieve, L., and Watson, C. J., *J. Lab. and Clin. Med.*, 1951, v37, 843.

11. Sveinsson, S. L., Rimington, C., and Barnes, H. D., *Scand. J. Clin. and Lab. Invest.*, 1949, v1, 2.

12. Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, v168, 133.

13. Watson, C. J., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 393.

14. Schwartz, S., and Wikoff, H. M., *J. Biol. Chem.*, 1952, v194, 563.

15. Watson, C. J., Lowry, P. T., Schmid, R., Hawkinson, V. E., and Schwartz, S., *Trans. Assn. Am. Phys.*, 1951, v64, 345.

16. Watson, C. J., De Mello, R. Pimenta, Schwartz, S., Hawkinson, V., and Bossenmaier, I., *J. Lab. and Clin. Med.*, 1951, v37, 831.

17. Sumegi, S., and Putnoky, J., *Arch. f. Gewerbe. Path.*, 1939, v9, 566.

18. Seggel, K. Ad., *Erg. Inn. Med.*, 1940, v58, 582.

19. Garrod, A. E., *Inborn errors of metabolism*, Henry Fronde, London, 1923.

20. Nicholas, R. E., and Rimington, C., *Biochem. J.*, 1951, v48, 306.

21. Fischer, H., and Zerweck, W., *Zeit. f. physiol. Chem.*, 1924, v137, 176.

Received January 14, 1952. P.S.E.B.M., 1952, v79

Ineffectiveness of Lyxoflavin as an Antithyrotoxic Factor for the Rat.
(19414)

BENJAMIN H. ERSHOFF.

From the Emory W. Thurston Laboratories, Los Angeles, Calif.

Available data indicate that water-insoluble liver residue contains a factor(s) distinct from any of the known nutrients (including vit. B_{12}) which is effective in prolonging survival and counteracting the growth retardation and gonadal inhibition of immature rats fed massive doses of thyroid in conjunction with a purified ration containing casein as the dietary protein and sucrose as the dietary carbohydrate(1-4). Recent findings by Emerson and Folkers(5) indicate that lyxoflavin was as effective as liver residue in promoting growth in hyperthyroid rats fed a soybean-meal-dextrose-containing diet. In the present communication data are presented on the comparative effects of lyxoflavin and liver residue on the gain in body weight, survival rate and gonadal weight of immature hyperthyroid rats fed a purified casein-sucrose-containing ration.

Procedure. The basal ration employed in the present experiment consisted of sucrose, 73.0%; casein,* 22.0%; salt mixture,† 4.5%; and U.S.P. desiccated thyroid,‡ 0.5%. To each kg of the above were added the following synthetic vitamins: thiamine hydrochloride, 72 mg; riboflavin, 9 mg; pyridoxine hydrochloride, 15 mg; calcium pantothenate, 67.2 mg; nicotinic acid, 60 mg; 2-methylnaphthoquinone, 5 mg; and choline chloride, 1.2 g. Each rat also received 3 times weekly the following supplements: cottonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg and a vit. A-D concentrate§ containing 50 U.S.P. units of vit. A and 5 U.S.P. units of vit. D. In addition to the basal ration the following diets were also employed, consisting of basal ration plus each of the following supplements: 1) 30 mg lyxoflavin‖/kg of diet 2) 100 mg lyxoflavin/kg of diet, and 3) 10% liver residue powder,¶ added in place of an equal amount of sucrose. A control ration was also employed consisting of the basal ration with thyroid omitted and replaced by an equal amount of sucrose. Sixty female rats of the Long-Evans strain were selected at 22 to 25 days of age and an average weight of 45.9 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to minimize access to feces and were fed the above diets *ad libitum* (20 rats on the basal ration; 10 rats on each of the other diets). After 60 days of feeding surviving animals were autopsied, and ovarian, adrenal and ventricular weights determined. Results are summarized in Table I.

Results. In agreement with earlier findings body and gonadal weight and length of survival were significantly reduced in thyroid-fed rats on the purified basal ration, whereas the ratio of adrenal and ventricular weight per 100 g body weight was increased, Supplements of lyxoflavin at a level of 30 mg or 100 mg per kg of diet were without significant effect either on gain in body weight, survival rate, ovarian weight or the ratio of adrenal and ventricular weight per 100 g body weight. Liver residue, on the other hand, completely counteracted the retardation in body and gonadal weight, increased the survival rate, and significantly reduced the ratio of adrenal and ventricular weight per 100 g body weight. It is apparent from these findings that under

---

* Vitamin Test Casein, General Biochemicals, Inc.

† Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc.

‡ Thyroid Powder, U.S.P., Armour and Co.

§ Nopco Fish Oil Concentrate, assaying 800000 U.S.P. units of vit. A and 80000 U.S.P. units of vit. D per g.

‖ We are indebted to Dr. Karl Folkers of Merck and Co. for the crystalline lyxoflavin employed.

¶ Extracted liver Residue (Lot No. 72638), Wilson Laboratories. This liver fraction consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances. Considerable variation has been observed in this laboratory in the antithyrotoxic activity of different batches of commercially available liver residue.

TABLE I. Comparative Effects of Lyxoflavin and Liver Residue on Gain in Body Weight, Survival Rate and Organ Weights of Immature Hyperthyroid Rats (20 Rats on the Basal Ration; 10 Rats on Each of the Other Diets).

Supplements fed with basal ration	Initial body wt, g	Avg gain in body wt, g, on:		Surviving, %	Ovarian wt, mg	Adrenal wt, mg	Adrenal wt, mg/100 g body wt	Ventricular wt, mg	Ventricular wt, mg/100 g body wt
		28th day	60th day						
None	46.4	52.3 (12)	67.5 (8)	40	21.4	47.1	41.7	850	752
Lyxoflavin, 30 mg/kg of diet	46.1	56.8 (7)	75.7 (5)	50	21	51.2	41.8	982	801
Lyxoflavin, 100 mg/kg of diet	46.6	53.4 (7)	81.5 (2)	20	28.6	64.8	48.3	980	731
Liver residue	44.8	101.6 (10)	159.4 (8)	80	54.6	59.6	26.6	958	467
Basal ration without thyroid	45.2	90.2 (10)	152 (10)	100	47.3	44.5	22.9	658	338

Values in parentheses indicate number of animals which survived and on which averages are based.

conditions of the present experiment the antithyrotoxic activity of lyoflavin was not equivalent to that of liver residue. No data are available to indicate what factors are responsible for the diverse results obtained by Emerson and Folkers(5) and the present investigator. It is well established, however, that differences in the carbohydrate, protein and fat components of a diet exert a profound effect on the nutritional requirements of the hyperthyroid rat(6). The above investigations differed not only in the carbohydrate, protein and fat components of the diet but in the mineral and vitamin content as well. Differences also existed in the sex and strain of experimental animal employed. These differences may have contributed to the diverse results reported above.

Summary. Experiments were conducted on the comparative effects of lyxoflavin and liver residue on the gain in body weight, survival rate and ovarian weight of immature hyperthyroid rats fed a purified ration containing casein as the dietary protein and sucrose as the dietary carbohydrate. Liver residue prolonged survival and completely counteracted the retardation in body and gonadal weight observed in thyroid-fed rats on the purified basal ration. Lyxoflavin was without significant effect.

---

1. Ershoff, B. H., *Arch. Biochem.*, 1947, v15, 365.

2. ———, Proc. Soc. Exp. Biol. and Med., 1950, v73, 282.

3.———, Proc. Soc. Exp. Biol. and Med., 1950, v73, 459.

4. ———, *Arch. Biochem.*, 1950, v28, 359.

5. Emerson, G. A., and Folkers, K., *J. Am. Chem. Soc.*, 1951, v73, 2398.

6. Ershoff, B. H., *Nutrition Fronts in Public Health*, Nutrition Symposium Series No. 3, 1951; The National Vitamin Foundation Inc., New York, 23.

Received January 15, 1952. P.S.E.B.M., 1952, v79.

Effect of Soya “Lecithin” on Number of Clostridia in Rat Feces. (19415)

F. W. Bernhart, G. T. Durbin, Elizabeth Linden, J. B. Hassinen, and
R. M. Tomarelli.

From the Research Laboratories, Wyeth Inc., Mason, Mich.

Recent studies with farm animals(1) and rats(2) have indicated that the putrefactive microorganisms of the intestinal flora may be undesirable. The dietary inhibition of putrefactive intestinal microorganisms has been accomplished by feeding lactose and dextrins

(3), low levels of protein(4), and by addition of antibiotics to the feed(1). Voet(5) reported that administration of lecithin to patients caused a decrease in odor of the feces. After confirming the observation in this laboratory by feeding soya "lecithin" to normal human subjects a study was made with rats to find out if the decrease in odor was due to changes in the intestinal flora. The present report describes an effect of soya phosphatides in reducing the number of clostridia in rat feces.

Experimental. Rats of the Sprague-Dawley strain maintained at constant temperature (25°C) in individual cages were fed *ad lib.* Twice a week, the diets were supplemented with 2 drops of oil concentrate containing vit. A (15,000 u), vit. D (2200 u), and α-tocopherol (10 mg) per g. Freshly dropped feces from individual rats were picked from blotting paper pads placed under the wire bottom of the cage. One g samples were added to 99 ml of sterile distilled water contained in dilution bottles and shaken mechanically for at least a half hour. From this $1\text{-}10^2$ dilution further dilutions of $1\text{-}10^4$ and $1\text{-}10^6$ were made and plated on Wilson-Blair(6) medium. This medium has been used for the estimation of *C. perfringens* in water(6), soils(7), wounds(17), and feces(1). One ml of the dilution was pipetted into a Petri dish and thoroughly mixed with 15-20 ml of the warm dextrose-sulfite-ferric chloride agar. After the agar had hardened, a thin top layer of dextrose agar was poured. After 20 hours of anaerobic incubation at 35°C, the black colonies were counted. Brewer Jars containing 90% N_2 and 10% CO_2 were used for maintenance of anaerobiosis. The crude soya "lecithin"* containing 2/3 crude phosphatides and 1/3 soybean oil was homogenized into skim milk supplying an equal weight of solids and spray dried; hence the dried "lecithin" compound used in the preparation of diets contained 50% skim milk solids, 33% phosphatides and 17% soybean oil.

Results. In a preliminary experiment, the soya "lecithin" 50% skim milk 50% compound was fed at a 25% level in a Rockland Rat Diet.† After 6 and up to 24 days on the "lecithin" diet the average Wilson-Blair plate count (5 rats, 30 determinations) per g of feces was 8,000; the range was from negative plates at 1-100 dilution to a maximum of 38,000. Parallel determinations on the control diet averaged 12 million with a range from negative at 1-100 dilution to a maximum of 159 million. The feces of the "lecithin" fed group contained on the average only about 1-1000th of the number of clostridia present in the feces of the control group. However, the low counts present in the feces of some members of the control group obscured the significance of the data and so control diets were sought which would result in high count in all rats.

A diet of horsemeat was found to increase consistently the Wilson-Blair plate counts of the feces from 3-30 (avg 13) millions per g(4). Addition of 12.5% skim milk powder to the horsemeat increased the fecal counts to higher levels 30-90 (avg. 57) million per gram. Addition of 12.5% soya "lecithin" to the skim milk-horsemeat diet reduced the clostridia counts after one week of feeding and during a period of 5 weeks (40 determinations on 5 rats) to an average of 200,000 colonies per g, approximately 1-300th of the control values.

To confirm these results, to find out about the reversibility of the effects, and also to obtain information about the constituents of soybean lecithin responsible for the decrease in clostridia, the following experiment was conducted. Forty male weanling rats were fed the horsemeat-skim milk diet for 24 days. The 30 rats having the highest fecal clostridia counts as determined by 2 counts during the last 2 weeks of this period were divided into 6 groups, 5 rats per group. Diets having the composition listed in Table I were then fed. From the data of Scholfield *et al.*(8) the amounts of choline and inositol supplied by diet L were calculated and these amounts were added in diets Ch, I, S, and M_1. Diets containing these components of "lecithin" phosphatides were tested because of their possible influence on intestinal flora. Inositol has

---

* A. E. Staley Co., Decatur, Ill.

† Arcady Farms Milling Co., Chicago, Ill.

TABLE I. Composition of Diets Fed (g/kilo).

	C	L*	S	Ch	I	M_1
Horsemeat	875	750	833	872	872	827
Skim milk solids	125	125	125	125	125	125
Crude phosphatides	—	83	—	—	—	—
Soybean oil	—	42	42	—	—	42
Choline	—	—	—	2.75	—	2.75
Inositol	—	—	—	—	3.25	3.25

* Diet prepared by using 25% "lecithin" compound described in text.

TABLE II. Clostridia Count of Feces of Rats Fed Various Diets (Millions/g of Feces).

Dates		Group No.					
Diet* changes	Determinations	1	2	3	4	5	6
8/27		C	C	C	C	C	C
	9/12	34	16	40	22	9	43
	20	26	44	20	38	51	17
	Avg	30	30	30	30	30	30
10/2		C	L	S	Ch	I	M_1
	10/9	93	95	95	182	130	60
	16	115	4.6	71	88	86	76
	23	22	1.2	1.7	23	60	37
	30	100	.006	11	14	114	13
	11/6	65	.13	74	44	126	44
	13	10	.004	31	15	72	2.7
	20	44	.016	13	19	57	11
11/22		C	C	M_1	M_1	M_1	L
	11/27	66	47	42	38	42	.3
	12/3	39	33	35	46	59	.014
	12	34	81	79	76	55	.91
12/12		C	L	L	L	L	L
	18	25	.27	.47	.23	.24	.01
	26	35	.006	.20	.004	.012	.83
12/27			C	C	C	C	C
	1/7		43	58	48	58	56

* Composition of diets listed in Table I.

been shown to change intestinal flora(12). The average fecal plate counts of the rats obtained as the result of feeding these diets are shown in Table II.

Inspection of data of Table II shows that only diet L containing soya phosphatides caused a significant reduction of the fecal clostridia counts. The average number of clostridia organisms per g in the feces of rats fed diet L (250,000) was about 1-200th of the average number present in the feces of the control group (47 million). In 90 determinations of the feces of "lecithin"-fed rats negative plates at 1-100 dilution occurred 22 times. The highest counts observed were 3 and 4 million, both occurring in one rat of Group 6. In the other 24 rats no values over 1 million were observed. The effect of dietary soya phosphatides in reducing the fecal clostridia count appeared as early as the 5th day of feeding, reached maximum after 2 weeks and was maintained during administration for as long as 30 days. The effect was reversible; removal of the phosphatides from the diet caused the fecal counts to rise to control values.

In a continuation of the study it was found that addition of ethanolamine either to the control diet (11 g/kg) or to diet M_1 (2.7 g/kg) resulted in no change of fecal clostridia counts from control values. Colonies picked at random from plates from both the control and "lecithin" fed groups when inoculated into milk resulted in stormy fermentation in 98 out of 135 trials. This result indicates the predominating species present was *C. perfringens*(9).

Attempts to demonstrate an *in vitro* effect of soybean "lecithin" phosphatides on clostridia cultures were unsuccessful as were attempts to demonstrate an inhibitory factor towards clostridia organisms in the feces of the rats fed soya "lecithin." No significant differences in the pH of the feces of the rats on the different diets was observed.

Studies were also made of the effect of the diets on the numbers of fecal coliform and proteus organisms. The number of fecal coliform organisms was estimated by EMB media plate counts(10). The average fecal EMB counts on the "lecithin" diet (0.8 million/g) were about ¼ of the average counts (3 millions/g) on the control diet. Estimations of the numbers of proteus in the feces were made with ricinoleic acid media(11). The average number of proteus per g in the feces of the control group and "lecithin" group was 25000 and 85000 respectively.

Discussion. The addition of penicillin and terramycin to pig and turkey diets results in increased growth accompanied by a great reduction of the number of clostridia organisms in the cecal contents and feces(1). Clostridia have been implicated as the cause of enterotoxemia in sheep(12), "6 day disease" of chicks(13), and gastrointestinal disease in humans(14). Clostridia are present in the feces of artificially fed infants in large numbers but are absent in the feces of breast fed children(15). Clostridia counts are higher in older adults(16). Administration of soybean "lecithin" with a consequent long lasting reduction of the numbers of intestinal clostridia may be a method for evaluating the effect of these organisms on growth and in disease.

Summary. The feces of rats fed a diet of horsemeat or horsemeat and dried skim milk, regularly contained a number of anaerobic micro-organisms which form black colonies on Wilson-Blair medium plates. These organisms were presumed to be mainly clostridia, especially *C. perfringens*. Addition of a soybean "lecithin" compound (soybean "lecithin" homogenized in skim milk and dried) supplying 8.3% phosphatides to a horsemeat-skim milk diet greatly reduced the clostridia count of the feces. Under the same conditions, the addition to the diet of soybean oil, choline, inositol or ethanolamine singly or in combination at the same levels supplied by the soybean "lecithin" compound resulted in no significant change in the number of these organisms. Therefore, the reduction in fecal clostridia count observed on addition of soybean lecithin to the diet was probably due to one or more of the soybean phosphatides.

---

1. Sieburth, J. McN., Gutierrez, J., McGinnis, J., Stern, J. R., and Schneider, B. H., Proc. Soc. Exp. Biol. and Med., 1951, v76, 15.

2. Gyorgy, P., Stokes, J., Smith, W. H., and Goldblatt, H., *Am. J. Med. Sci.*, 1950, v220, 6.

3. Bergeim, O., *J. Biol. Chem.*, 1924-25, v52, 49.

4. Cannon, P. R., *J. Inf. Dis.*, 1921, v29, 369.

5. Voet, Quoted in "Lecithin", R. Kunze, Edwards Bros., Ann Arbor, 1945.

6. Wilson, W. J., and Blair, E. M. M'V., *J. Path. Bact.*, 1924, v27, 119.

7. Skinner, C. E., and Baskin, A. H., Proc. Soc. Exp. Biol. and Med., 1932, v29, 551.

8. Scholfield, C. R., Dutton, H. J., Tanner, F. W., Jr., and Cowan, J. C., *J. Am. Oil Chem. Soc.*, 1948, v25, 368.

9. Breed, R. S., Murray, E. G. D., and Hitchens, A. P., Bergey's Manual of Determinative Bacteriology, 6th Ed., 1948, Williams and Wilkins, Baltimore.

10. *Standard Methods for Examination of Dairy Prod.* 9th Ed., Am. Pub. Health Assn., New York, 1948.

11. Zarett, A. J., Doetsch, R. N., and Hansen, P. A., *J. Bact.*, 1949, v57, 266.

12. Merchant, I. A., *Veterinary Bacteriology*, 3rd. Ed., Ames, Iowa State College Press, 1946.

13. Lindley, D. C. and Kunha, T. J., *J. Nut.*, 1946, v32, 47.

14. McGaughey, C. A., *Vet. Rec.*, 1944, v56, 508.

15. Morris, G. B., Porter, R. L., and Meyer, K. F., *J. Inf. Dis.*, 1919, v25, 349.

16. Olsen, E., *Studies on the Intestinal Flora of Infants*, Copenhagen, Ejnar Munksgaard, 1949.

17. Lyons, C., and Owens, C. R., *J. Bact.*, 1942, v43, 685.

Received January 31, 1952. P.S.E.B.M., 1952, v79.

Respiration and Anaerobic Glycolysis of Transplanted Cartilage.* (19416)

Daniel M. Laskin, Bernard G. Sarnat, and James A. Bain.
From the Departments of Oral and Maxillofacial Surgery, Surgery and Pharmacology, Colleges of Dentistry and Medicine, University of Illinois, Chicago.

Current knowledge concerning the viability of costal cartilage following transplantation has been derived primarily from static gross and histologic studies. Reports on the metabolism of these grafts are entirely lacking. The present investigation was therefore undertaken in order to study and compare the respiration and anaerobic glycolysis of fresh costal cartilage with that transplanted to another area in the same animal (autografts), as well as between different animals of the same species (homografts).

Material and methods. Non-littermate, female albino rabbits, approximately 3 months of age and 2 kg in weight, were selected for these studies. Costal cartilage was removed from the donor rabbits under aseptic conditions, carefully stripped of perichondrium and embedded subcutaneously in the abdominal wall of the recipient animals (53 autografts and 53 homografts). The grafts were removed at intervals of 7, 11, 15, 30, 60, 90, 120, and 150 days following transplantation. Generally, at least 3 transplants were tested at each of the specific time periods. A series of 20 determinations on costal cartilage immediately following its removal from donor animals served for the comparison of fresh with transplanted tissue. Respiration and anaerobic glycolysis were studied by standard manometric methods. After the removal of any adherent connective tissue, the cartilage was sliced in cross-section to a thickness of 0.3 mm by means of a modified microtome and approximately 250 mg was placed in each flask. The suspending medium for the tissue slices in the aerobic determinations consisted of 2.0 ml of Krebs-Ringer phosphate solution, 0.2 ml of 0.001 M aqueous methylene blue, and 0.2 ml of 10% glucose. For the absorption of carbon dioxide, 0.2 ml of 2 N KOH together with a small piece of fluted filter paper were placed in the center well of each flask. The manometers were gassed for 10 minutes with pure oxygen bubbled through water. The suspending medium for the anaerobic investigations consisted of 2.0 ml of Krebs-Ringer bicarbonate solution, 0.4 ml of distilled water, and 0.2 ml of 10% glucose. The manometers were gassed for 10 minutes with a water saturated mixture of 95% nitrogen and 5% carbon dioxide. The flasks were maintained at approximately 38°C. After the manometers were allowed to equilibrate for 15 minutes, readings were taken at 15-minute intervals over a 2-hour period. Oxygen consumption (Q_{O_2}) and anaerobic glycolysis ($Q_G^N 2$) were expressed in terms of microliters per hour per mg of dry tissue.

Results. The results obtained from the study of respiration and anaerobic glycolysis of fresh costal cartilage appear in Table I. Because of the low rate of metabolic activity of the cartilage slices, it was found necessary to read the manometers at prolonged intervals so as to record significant variations. Moreover, since the oxygen uptake of the tissue was quite small, 0.2 ml of 0.001 M aqueous methylene blue was added to each aerobic system in order to increase further the magnitude of the manometric changes.

In the 20 determinations on fresh cartilage

TABLE I. Comparison of Respiration and Anaerobic Glycolysis of Fresh and Transplanted Rabbit Costal Cartilage.*

Costal cartilage	No. of specimens	Mean Q_{O_2}	No. of specimens	Mean $Q_G^N 2$
Fresh	10	−.41 ± .04	10	+.70 ± .08
Autograft	25	−.23 ± .08	28	+.46 ± .12
Homograft	28	−.22 ± .07	25	+.40 ± .13

* The mean Q_{O_2} and $Q_G^N 2$ values for autografts and homografts differed from the values of fresh cartilage at less than 1% level of probability.

---

* This investigation was supported by a research grant from the National Institutes of Health, U. S. Public Health Service, and by a grant from the Graduate College Research Board of the University of Illinois.

from different rabbits, the mean Qo_2 was found to be -0.41 ± 0.04 while the Q_G^N2 figure was $+0.70 \pm 0.08$. The fresh cartilage slices were found to retain approximately 32% of their weight following drying. After implantation for 7 days, the mean rate of respiration for the autografts was -0.26 while the value for the homografts was -0.32. From 7 to 150 days after transplantation the mean Qo_2 values for the autografts varied between -0.13 and -0.29. In the homografts, the rates ranged from -0.16 to -0.28.

The mean Q_G^N2 values were consistently higher than the corresponding Qo_2 figures. After the 7-day implantation period, the mean rates of anaerobic glycolysis for the autografts and homografts were $+0.50$ and $+0.54$ respectively. During the interval from 7 to 150 days the values varied from $+0.34$ to $+0.57$ for the autografts and $+0.29$ to $+0.50$ for the homografts.

Upon drying, the autografts retained $32.3 \pm 1.6\%$ of their weight, while the homografts retained $32.9 \pm 3.1\%$. Because of the similarity of the figures, 32% was used as the representative value for the entire series.

Discussion. Fresh cartilage possesses an extremely low rate of metabolism(1-7). The present study corroborated this finding. Following transplantation, the rates of respiration and anaerobic glycolysis were even lower than those of fresh cartilage. At the end of the first 7 days a marked decrease was noted in all of the grafts. From 7 to 150 days, however, statistical analysis revealed no significant difference between the values for consecutive time periods in either the autografts or homografts. The results in each series were therefore grouped irrespective of the time elapsed since implantation (Table I). Comparison of the collective values revealed that there was no salient difference between the metabolism of cartilage autografts and homografts in the rabbit (t value 0.50 for Qo_2, 1.75 for Q_G^N2; 53 degrees of freedom).

The fact that the autogenous and homogenous transplants gave well-defined values for both respiration and anaerobic glycolysis denoted the presence of living chondrocytes. This substantiated the previous gross and histologic evidence that both types of grafts remained in a viable state following transplantation(8-10). The relatively higher Q_G^N2 values recorded from these implants indicated, moreover, that the carbohydrate metabolism of both fresh and transplanted cartilage was predominantly anaerobic. This was in contrast to the findings reported for most other tissues.

The lowered Qo_2 and Q_G^N2 values displayed by these grafts could be partially attributed to a decline in the metabolism of the chondrocytes following transplantation. The decrease in the metabolic activity per cell, however, was not actually as great as the data would seem to indicate, because the Qo_2 and Q_G^N2 values were computed from the weight of the graft without consideration of its viable cell content. In a firm structure such as cartilage cellular necrosis can occur without significant weight loss since the matrix remains relatively intact. Thus, two grafts of equal weight could have entirely different viable cell counts and, while the metabolic quotients obtained from each would be different, the metabolism of any cell in either graft could be the same. In this investigation a decrease in the number of vital chondrocytes probably occurred in all of the grafts not only from the changes in metabolic activity during and after transplantation but also from the trauma incident to the surgical handling of the tissue. This decrease, therefore, would account for at least a portion of the diminished metabolism recorded from the cartilage grafts.

Summary. 1. The rate of respiration and anaerobic glycolysis of fresh rabbit costal cartilage is among the lowest of all tissues. Carbohydrate metabolism is predominantly anaerobic. 2. Transplantation resulted in approximately a 45% decline in the rates of both respiration and anaerobic glycolysis in both the autografts and homografts during the first 7 days. The major portion of the metabolic activity, however, remained on an anaerobic basis. 3. After 7 days no significant variations occurred in either the Qo_2 or Q_G^N2 values for any of the grafts. 4. No salient difference was observed between the metabolic activity of costal cartilage autografts and homografts in the rabbit from 7 to 150 days after transplantation.

---

1. Kuwabara, Gitaro, *J. Biochem.*, 1932, v16, 389.
2. Bywaters, E. G. L., *Nature*, 1936, v138, 30.
3. Dickens, F., and Weil-Malherbe, H., *Nature*, 1936, v138, 125.
4. Bywaters, E. G. L., *Nature*, 1936, v138, 288.
5. ———, *J. Path. and Bact.*, 1937, v44, 247.
6. Hills, G. M., *Biochem. J.*, 1940, v34, 1070.
7. Rosenthal, O., Bowie, M. A., and Wagoner, G., *J. Cell. and Comp. Physiol.*, 1941, v17, 221.
8. Dupertuis, S. M., *Arch. Surg.*, 1941, v43, 32.
9. Young, Forrest, *Surgery*, 1941, v10, 7.
10. ———, *Surgery*, 1945, v17, 616.

Received February 11, 1952. P.S.E.B.M., 1952, v79.

Inhibition of Utilization of Glutamic Acid by *Lactobacillus arabinosus*.* (19417)

PADMASINI AYENGAR† AND EUGENE ROBERTS.
From the Wernse Laboratory of Cancer Research, Washington University School of Medicine, St. Louis, Mo.

Patterns of free amino acids determined by 2-dimensional paper chromatography were studied in a variety of mouse tumors, comparing the neoplastic with normal homologous tissues(1,2). It was found that each normal tissue examined had a distribution of free amino acids characteristic for that tissue, while all of the tumors, regardless of origin or source, showed very similar patterns. One of the most interesting findings was that the glutamine levels in the tumors were uniformly lower than in most of the normal tissues. It appeared feasible to attempt to disturb the protein synthetic mechanisms in tumors by interference with glutamine metabolism, since glutamine may play a strategic role in protein synthesis by virtue of its participation in amide exchange reactions(3-5). Most normal tissues, which have relatively large amounts of free glutamine, would presumably be more resistant to such an interference.

Glutamine metabolism could be disturbed by inhibiting the synthesis of glutamine or by blocking its subsequent utilization in further metabolic processes. *Lactobacillus arabinosus* 17-5 can serve as a good test organism for screening agents which can act in either manner, since this organism can be used both for glutamic acid and for glutamine assay. The present communication describes the results of the tests of several compounds in these assay systems. Methionine sulfoxide and β-hydroxyglutamic acid, previously reported antimetabolites for glutamic acid in *L. arabinosus*(6,7), and γ-glutamylhydrazide, a glutamine antagonist for some strains of hemolytic streptococci(8), have also been studied.

Experimental, assay methods. The essential microbiological procedures have all been described previously(9) and references to the original literature given. All glutamic acid assays were performed at pH 6.0 in a low aspartic acid medium(10) (Medium 1), conditions which minimize lag. In most instances the glutamine assays were performed at pH 7.0 in a high aspartic acid medium(11) (Medium 2), under which circumstances there is a sharp differentiation in response to glutamine and glutamic acid in the low concentration ranges. In other experiments the same conditions were used for glutamine as for glutamic acid. Solutions of all of the substances tested and glutamine were sterilized by Seitz filtration. When determination of growth was made at only one time interval, the turbidity readings were made at 24 hours. Several experiments were performed in which readings were made at numerous time intervals. All

---

* This investigation was supported by research grants from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, and the Charles F. Kettering Foundation.

† Supported by a fellowship from the Charles F. Kettering Foundation. Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

TABLE I. Inhibition of Growth by β-Hydroxyglutamic Acid† "A" and Its Reversal with L-Glutamic Acid (Medium 1).

Amt of test substance/tube,* γ	Optical density		Amt of glutamic acid/tube, γ	Optical density	
	BHG "A"	BHG "B"		No addition	500 γ of BHG "A"
0	.179	.213	0	.0	.0
100	.223	.237	10	.030	.0
200	.223	.226	20	.061	.0
300	.223	.257	30	.095	.0
400	.162	.255	40	.144	.008
500	.084	.251	50	.196	.019
600	.014	.240	60	.244	.123
900	.0	.280	90	.359	.310
1200	.0	.293	120	.445	.352
1500	.0	.255	150	.468	.396

* All tubes contained 50 γ of L-glutamic acid. † BHG.

readings were made in a room maintained at 37°, so that the tubes were at the same temperature during the entire experiment. *Compounds tested.* Nine of the compounds tested were kindly furnished by Dr. Karl Pfister of Merck and Co., Inc. Of these, L-methionine sulfoxide(12,6), L-γ-glutamyl hydrazide(8), DL-β-aspartyl hydrazide(8), L-γ-methylglutamate(13), and DL-pyrrolidone carboxylic acid(14) were secured by the indicated literature procedures. New synthetic methods, to be described in a later publication from the laboratories of Merck and Co., Inc.,[‡][§] were employed in the preparation of DL-α-methylglutamic acid (m.p. 168-70° decomp.); DL-β-hydroxyglutamic acid ("A" form, m.p. 198° decomp.; "B" form, as hydrate, m.p. 180-5° decomp.; anhydrous "B" form, m.p. 157-8° decomp.). (It is possible that a β-hydroxyglutamic acid reported to melt at 75° as a trihydrate and then to solidify and remelt at 185°(16) is identical with the "B" form of hydroxyglutamic acid mentioned above). The previously described γ-methylamide of L-glutamic acid(15) was kindly furnished by Dr. F. C. Meyer of the Research Department of the Monsanto Chemical Co.

Results. Results of initial screening tests. In the concentrations used (40 to 3000 γ per tube) only 2 of the substances were markedly inhibitory for growth on glutamic acid and none of them inhibited the utilization of glutamine. Methionine sulfoxide was not inhibitory under the present conditions of testing, even when the concentration present was 60 times that of glutamic acid. Although L-γ-glutamylhydrazide inhibits the utilization of glutamine in some streptococci(8), it did not inhibit the utilization of glutamine in the present test system. A more detailed description of the inhibitory properties of α-methylglutamic acid and β-hydroxyglutamic acid "A" will follow.

Inhibition of growth on glutamic acid by β-hydroxyglutamic acid "A". Table I shows the results of an experiment in which were tested the inhibitory effects of the racemates of the two diastereoisomers of β-hydroxyglutamic acid (BHG). The "A" acid was markedly inhibitory while the other isomer showed no inhibition and even possibly a slight stimulation of growth. It is seen that the inhibition is competitive with glutamic acid, since increasing amounts of glutamic acid in the presence of a constant amount of inhibitor decreased the extent of inhibition.

TABLE II. Inhibition of Growth by α-Methylglutamic Acid (All Tubes Contained 50 γ L-glutamic Acid; Medium 1).

Amt of α-methylglutamic acid per tube, γ	% of normal growth Exp. No. 1	2	3
100	56.2	92.7	55.5
200	37.5	71.1	54.5
300	16.3	18.7	48
400	6.9	3.9	—
500	5.9	2.3	34.9

[‡] Rogers, E. F., Conbere, J. P., Leanza, W. J., and Pfister, K.

[§] The authors are grateful to Dr. W. W. Umbreit of Merck and Co., for his interest which made possible the cooperation of the St. Louis and Rahway laboratories in this work.

TABLE III. Reversal of Inhibition by α-Methylglutamic Acid with L-Glutamic Acid (Medium 1).

Amt of glutamic acid/tube, γ	Optical density				
	Exp. 1		Exp. 2		
	Control	500 γ inhibitor/tube	Control	500 γ inhibitor/tube	1500 γ inhibitor/tube
10	.029	.0	.022	.0	—
20	.059	.0	.046	.0	—
30	.095	.011	.071	.006	.014
40	.143	.030	.120	.027	—
50	.192	.063	.168	.060	—
60	.244	.108	.219	—	.116
90	.357	.254	.343	—	.264
120	.445	.399	.432	—	.371
150	.468	.466	.458	—	.421

Inhibition of growth on glutamic acid by α-methylglutamic acid. In Table II are the results of 3 different experiments showing the inhibitory effects of different concentrations of α-methylglutamic acid (AMG) on growth in the presence of 50 γ of glutamic acid. Although inhibition was found in each experiment, the extent of inhibition differed from one experiment to the other. This was also found to be the case in a number of other similar experiments not reported in detail. A possible explanation for this will be suggested in a subsequent section. Increasing concentrations of glutamic acid produced a reversal of the inhibition exerted by a given amount of AMG (Table III), showing the competitive nature of the inhibition.

Time-course of growth on glutamic acid in presence and absence of AMG. In Fig. 1 is shown the 3-dimensional plot of the time-course of growth with glutamic acid alone under the standard assay conditions, employing time, optical density, and glutamic acid concentration as the variables. It is seen that little additional growth occurred after 24 hours at the lower concentrations and that suitable growth curves for assay purposes were obtained between 22 and 30 hours. In Fig. 2 are plotted the results of an experiment similar in all respects to that shown in Fig. 1 with the exception that all tubes contained 1500 γ of AMG. A comparison of the results in Fig. 1 and 2 shows clearly the marked inhibitory effect of the AMG. However, the inhibition was not absolute, since there was some growth in the presence of inhibitor at 41 hours in the tubes containing the 4 highest concentrations of glutamic acid. Although the growth with 150 γ of glutamic acid in the presence of AMG was only 28% of the control at 24 hours, it was 75% of the normal value at 30 hours. Similarly, in the presence of 120 γ of glutamic acid the inhibited growth was 9% of the control at 24 hours, but 59% at 30 hours. It is evident from this that although readings taken at only one time interval may give good qualitative evidence of the inhibitory potentialities of a compound in the present test series, little quantitative significance can be attached to such results. Inhibition ratios calculated from such data would be of little value. The variability observed in the degree of inhibition found for a given amount of AMG when growth was measured at 24 hours (Table II) could possibly have been caused by different rates at which the inhibition was overcome in the individual experiments.

Mechanism of action of α-methylglutamic acid and β-hydroxyglutamic acid "A". Results with methionine sulfoxide and an unresolved mixture of BHG indicated that these substances were active by virtue of their ability to prevent the amidation of glutamic acid(6,7). Small amounts of glutamine were able to overcome the inhibition, and the utilization of glutamine itself was not appreciably affected. The same mechanism is probably operative in the present experiments with AMG and BHG. Neither compound inhibited the utilization of glutamine. Actually there may have been a slight stimulation by the AMG and BHG "A", both of which were strongly inhibitory in the utilization of glu-

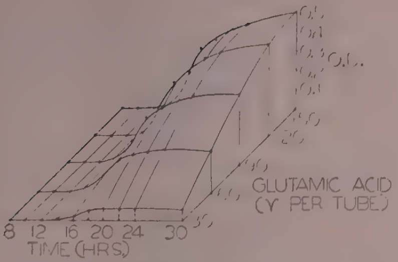


FIG. 1. Time-course of growth on different amounts of L-glutamic acid.

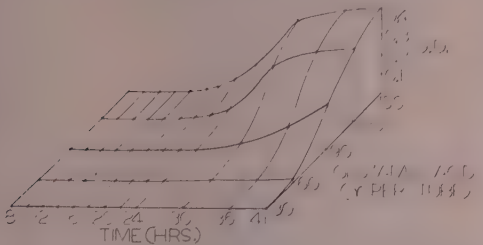


FIG. 2. Time-course of growth on different amounts of L-glutamic acid in the presence of 1500 γ of AMG per tube.

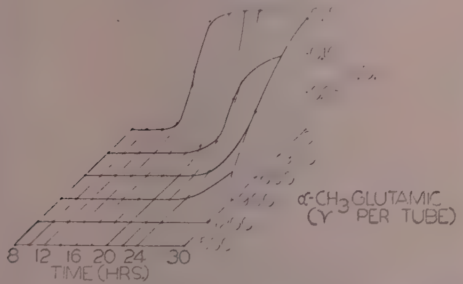


FIG. 3. Time-course of growth on 50 γ of L-glutamic acid in the presence of varying amounts of AMG.

tamic acid. In an experiment in which the time-course of growth was followed it was found that there was no growth for 18 hours when amounts of AMG from 300 to 1500 γ per tube were used in the presence of 50 γ of glutamic acid (Fig. 3). At this time the control tubes had attained almost maximal growth. However, at 30 hours the tubes containing 300 γ of AMG had 86% of the growth found in the controls, and the tubes containing 600 and 900 γ of AMG had begun to show a rapid growth. This emphasizes the importance of the consideration of the time factor in the reversal as well as the inhibition studies. It is doubtful in the present instance that the inhibition is overcome because of the destruction of the inhibitor. It would rather appear that there is a very slow rate of synthesis of glutamine going on. When a small critical concentration of glutamine has been attained the utilization of glutamic acid may proceed independently of the ability of the organism to synthesize glutamine.

Influence of α-methylglutamic acid on the time-course of growth on glutamine alone and on a mixture of glutamine and glutamic acid. The standard growth curves for glutamine were very similar to those found for glutamic acid (Fig. 1) when assays were performed in Medium 1. When varying amounts of AMG were added to 50 γ of glutamine virtually identical growth curves were obtained (Fig. 4). No increased time-lag in growth was noted at any concentration of inhibitor tested. There was a slight depression in the extent of growth below that in the control tubes in all of the samples containing AMG, but this was the same regardless of the concentration of AMG. A comparison of Fig. 3 and 4 shows clearly the remarkable difference between the effect of AMG on the utilization of glutamic acid and glutamine. It was previously suggested that AMG acts by preventing the synthesis of glutamine from glutamic acid, but that once small amounts of glutamine become available the bacteria can utilize the remainder of the glutamic acid. From this it would be expected that small amounts of glutamine should enable growth to take place on small quantities of glutamic acid in the presence of

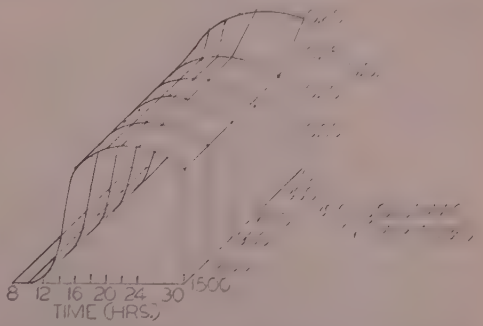


FIG. 4. Time-course of growth on 50 γ of L-glutamine in the presence of varying amounts of AMG.

a large amount of inhibitor. An experiment was performed in which each tube contained a mixture of 35 γ of glutamic acid and 15 γ of glutamine in the presence of varying amounts of AMG up to 1500 γ per tube. When 50 γ of glutamic acid alone was used in the presence of 1200 γ and 1500 γ of AMG per tube, there was no growth at all in 30 hours (Fig. 3). In the present experiment there was visible growth in all tubes by 16 hours. Even in the tubes containing 1200 γ and 1500 γ of AMG growth amounting to 89% and 78% of normal was found at 30 hours, showing clearly the marked protection afforded by small amounts of glutamine. However, some inhibition of growth by AMG was noted in the earlier time periods. It may well be that AMG also inhibits some steps in glutamic acid utilization other than amidation.

Inhibition of the α-ketoglutarate-glutamine system. It was shown previously that α-ketoglutarate does not replace glutamine or glutamic acid for growth in Medium 2(9). Good growth occurred with α-ketoglutarate in the presence of small amounts of glutamine. The results suggested that the role of glutamine is catalytic. It was of interest to see whether this system could be inhibited by some of the substances tested for glutamic acid antagonism. From the results in Table IV it is seen that methionine sulfoxide and AMG are weakly inhibitory and that BHG "A" appears to be a somewhat stronger inhibitor. These findings are being extended in further studies.

TABLE IV. Inhibition of the α-Ketoglutarate–Glutamine System (All Tubes Contained 2.5 mg α-Ketoglutaric Acid and 30 γ of Glutamine; Medium 2).

Amt of inhibitor /tube, γ	Optical density		
	Methionine sulfoxide	α-Methylglutamic acid	β-Hydroxyglutamic acid A
0	.309	.301	.333
100	—	.330	.231
200	—	.290	.215
300	.288	.297	.210
400	—	.286	.194
500	—	.261	.171
600	.286	—	—
900	.268	—	—
1200	.254	—	—
1500	.223	—	—
1800	.244	—	—
2400	.222	—	—
3000	.210	—	—

Discussion. The purpose of the present investigation was to find inhibitors of glutamic acid and glutamine utilization in a microbiological test system which would also be effective in mammalian tissues. α-Methylglutamic acid (AMG) and one of the diastereoisomeric racemates of β-hydroxyglutamic acid were more potent inhibitors of glutamic acid utilization for *L. arabinosus* than any compounds previously described. All of the experiments performed were consistent with the interpretation that these compounds acted by preventing the synthesis of glutamine from glutamic acid. Tests are in progress to determine whether the amidation of glutamic acid in animal tissues is also inhibited by these substances. Preliminary tests with mice of the $C57_6$ strain weighing between 14 and 18 g have shown that these animals can tolerate for at least 10 days 30 mg of AMG administered daily by the intraperitoneal route without showing any toxic symptoms or loss in weight. An indication of the *in vivo* antagonism of AMG to glutamic acid also was obtained when it was found that this compound significantly enhanced the incidence of audiogenic seizures over the controls in a susceptible strain of mice (dba) tested under standard conditions(17). Marked reductions in seizure incidence and in fatalities below the control levels were obtained when glutamic acid was given. Tests are now being conducted with AMG in tumor-bearing animals. AMG has also been found to be a potent inhibitor of a bacterial glutamic acid decarboxylase and a somewhat weaker inhibitor of brain glutamic decarboxylase. These results will be reported subsequently.

Summary. 1. Ten compounds were tested for inhibition of utilization of glutamic acid and glutamine by *Lactobacillus arabinosus*. 2. DL-α-methylglutamic acid and one of the diastereoisomeric racemates of β-hydroxyglutamic acid were potent competitive inhibitors of glutamic acid utilization. None of the compounds tested was a glutamine inhibitor. 3. The results indicated that the two active compounds prevented the synthesis of glutamine from glutamic acid by the bacteria. 4. Experiments in which the time-course of growth was followed showed that under certain conditions the inhibition by α-methylglutamic acid could be overcome. A possible explanation was suggested for this finding and the importance of taking readings at several time intervals was emphasized.

---

1. Roberts, E. and Frankel, S., *Cancer Res.*, 1949, v9, 645.

2. ———, *Cancer Res.*, 1950, v10, 237.

3. Waelsch, H., Owades, P., Borek, E., Grossowicz, N., and Schou, M., *Arch. Biochem.*, 1950, v27, 237.

4. Grossowicz, N., Wainfan, E., Borek, E., and Waelsch, H., *J. Biol. Chem.*, 1950, v187, 111.

5. Stumpf, P. K., Loomis, W. D., and Michelson, C., *Arch. Biochem.*, 1951, v30, 126.

6. Waelsch, H., Owades, P., Miller, H. K., and Borek, E., *J. Biol. Chem.*, 1946, v166, 273.

7. Borek, E., and Waelsch, H., *J. Biol. Chem.*, 1949, v177, 135.

8. Roper, J. A., and McIlwain, H., *Biochem. J.*, 1948, v42, 485.

9. Ayengar, P., Roberts, E., and Ramasarma, G. B., *J. Biol. Chem.*, 1951, v193, 781.

10. Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Biol. Chem.*, 1949, v177, 533.

11. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, v172, 15.

12. Toennies, G., and Kolb, J. J., *J. Biol. Chem.*, 1939, v128, 399.

13. Hanby, W. E., Waley, S. G., and Watson, J., *J. Chem. Soc.*, 1950, Part 4, 3239.

14. Lichtenstein, N., and Grossowicz, N., *J. Biol. Chem.*, 1947, v171, 387.

15. Lichtenstein, N., *J. Am. Chem. Soc.*, 1942, v64, 1021.

16. Harington, C. R., and Randall, S. S., *Biochem. J.*, 1931, v25, 1917.

17. Ginsburg, B. E., and Roberts, E., Presented at the 118th Annual Meeting of the AAAS, Philadelphia, Dec. 26-31, 1951.

Received February 11, 1952. P.S.E.B.M., 1952, v79.

Virulence Enhancing Activities of Aureomycin on *Candida albicans*.*

(19418)

ERICH SELIGMANN. (Introduced by William Antopol.)

From the Division of Laboratories, Beth Israel Hospital, New York City.

Personal observations and a number of reports in the literature(1) indicate that proliferation of fungus-like organisms, notably *Candida albicans* may occur in patients treated with penicillin, aureomycin, chloramphenicol, and terramycin. Not only is there a numerical increase in the oral cavity, intestinal or urogenital tract, etc., but localized or disseminated disease with serious, and at times fatal consequences may develop.

The mechanism of stimulation of growth and increase in pathogenicity of fungi in patients treated with antibiotics is not fully understood. The frequency of these complications has become of sufficient impact to warrant the American Council on Pharmacy and Chemistry of the A.M.A. to issue "a warning statement to be included in aureomycin hydrochloride, chloramphenicol and terramycin hydrochloride labeling"(2). The Council suggests that susceptible bacteria are suppressed by the bacteriostatic action of the drugs, and monilia or other yeast-like organisms may then replace the normal or abnormal bacterial flora, thus paving the way for secondary mycotic infection. The following experiments may shed some light on these problems.

Material and methods. Candida albicans. A strain isolated from the oral cavity of a patient was used for all the tests. The strain grew in fluid media with sediment and no pellicle formation, fermented dextrose, maltose and sucrose, did not attack lactose. On Sabouraud's agar it produced white, creamy colonies, on blood agar plates dull grey colony forms and on corn meal agar streaks, it developed the characteristic mycelium and yeast cell clusters. It was therefore considered a typical *Candida albicans*. For animal tests, the growth from Sabouraud's agar slants was washed off with normal saline solution and centrifuged at 1500 r/min. The white sediment of packed cells was suspended in saline (0.1 ml cells + 2.4 ml saline). 0.1 ml of the cell sediment contained approximately 4 billion cells. The usual dose of 0.2 ml of the suspension corresponded to 320 million living cells. The animal injections were given intraperitoneally. *Aureomycin.* "Aureomycin crystalline intravenous Lederle", was used. The brown crystals come in sealed ampules containing 100 mg of the drug. 10 ml normal saline were introduced into the ampule and the contents dissolved. 1 ml of the yellow solution corresponds to 10 mg aureomycin, the dose of 0.2 ml normally used to 2 mg. Because of the known lack of stability of these solutions,[†] only freshly prepared ones were employed unless stated otherwise.

---

* This investigation was supported by the Joseph and Helen Yeamans Levy Foundation.

Experiments. The strain of *Candida* employed proved to be nonpathogenic for mice on intraperitoneal injection of large amounts of cells (320 million) in saline. Of 18 mice so treated all survived after 2 weeks. The aureomycin dose of 2 mg intraperitoneally was non-toxic. Although the animals became restive for 1-2 minutes after the obviously painful injection of the latter, all of the 17 mice so treated survived after 2 weeks. However when *Candida* cells were suspended in aureomycin solution (0.1 ml cells + 2.4 ml aureomycin) and the mixture injected intraperitoneally, 16/17 mice died. The following Table I (A-C) demonstrates the combined results of 3 experiments.

On 32 repeated administrations of the mixture, 28/32 mice died in 3-48 hours. No gross signs of peritonitis or involvement of organs (kidney, spleen, liver, adrenals) were seen. In each case, *Candida* was recovered on culture from the peritoneal cavity, and in 10 out of 27 on heart blood culture. In 3 mice there was complication of *S. enteritidis* infection and these animals were excluded from the evaluation. Numerous *Candida* strains recovered from dead mice were tested again in normal mice. None had acquired pathogenicity. When the aureomycin-*Candida* mixtures were centrifuged, and the supernatant and washed sediment injected separately in amounts corresponding to 0.2 cc of the original mixture, they proved non-toxic, Table I (D-F). There was thus no indication of an interaction between aureomycin and *Candida* organisms *in vitro*. In another group of experiments, aureomycin and *Candida* organisms were administered separately at different time intervals.

TABLE I. Action of Candida, Aureomycin, and of Their Mixture in Mice.

Mice group	Material injected, .2 ml	Mortality*
A	Candida cells in saline sol.	17/17
B	Aureomycin sol.	17/17
C	Mixture of Candida cells in aureomycin	1/17
D	Candida cells in aureomycin susp.	0/9
E	Supernate of Candida-aureomycin mixture after centrifugation	9/9
F	Washed Candida sediment	8/9

* Numerator: Survivors after 2 wk. Denominator: Total No. of mice.

TABLE II. Separate Injection of Aureomycin and Candida Cells in Mice.

Mice group	Treatment	Results*
A	Inj. 24 hr *before* infection	5/9
B	8	4/9
C	2	1/10
D	Simultaneous inj. and infection	1/10
E	Inj. 4 hr *after* infection	3/10
F	8	9/10
G	24	10/10

* Numerator: Survivors after 2 wk. Denominator: Total No. of mice.

Inj = i.p. injection of 2 mg aureomycin sol., infection = i.p. inoculation of .2 ml Candida suspension in saline.

From Table II it is apparent that when aureomycin is given from 24 hours before until 4 hours after infection with *Candida* there was a considerable mortality; the toll was higher as the aureomycin injection and *Candida* infection were administered at closer intervals. If, however, *Candida* cells were subjected to action of the mouse for 8 or 24 hours before administration of aureomycin, the increased pathogenicity did not occur. One may conclude that under these conditions the antifungal activities of the mouse tissues could play their part, while with reversed order of the injections aureomycin interfered for a considerable time with the normal defense mechanism of the animal.

[†] On standing, precipitation begins on the second day and considerable sediment is present in 3-4 days.

It is known that aureomycin solutions kept at icebox or room temperature for a few days lose considerable antibiotic potency. When such solutions were used in the *Candida* tests above reported, the virulence enhancing capacity was also lost. Ten mice were injected intraperitoneally with 0.2 ml each of a suspension of 0.1 ml packed cells in 2.5 ml aureomycin solution which has been kept at room temperature for two days: all 10 survived.

The antibiotic capacity of aureomycin in solution is heat labile. When the solution of crystals in saline was brought to the boiling point and then used for the "*Candida*-aureomycin mixture test", 13 of 14 mice injected intraperitoneally with 0.2 ml of the mixture survived. It is apparent therefore, that a parallelism exists between antibiotic (against bacteria) and virulence enhancing (for *Candida*) faculties of aureomycin solutions.

Discussion. The problem of fungus proliferation *in vivo* under antibiotic influence is not only one of survival in favorable conditions but also one of activation. It can not be explained by the suppression of sensitive bacteria alone and consequent lack of competition for nutritive substances or by induced suppression of growth-restraining factors dependent upon the elimination of the customary bacterial flora. Direct growth-promoting action of the antibiotics as well as changes of inherent pathogenicity of the fungi might be involved. Most antibiotics are known to promote growth of animals(3). That the effect is not necessarily due to control of the intestinal flora was stressed by Weber *et al.*(4). Elam and co-workers(5) reported that parenteral application of penicillin and bacitracin while not influencing the fecal flora at all was able to promote growth in the chick.

Although some workers have found a direct growth-promoting action of antibiotics on fungus cells *in vitro*, the results in other instances have been inconclusive or negative (6-9). In our own experiments, aureomycin in ordinary and synthetic liquid media or on nutrient agar slants has failed to promote the growth of *Candida albicans*. The possibility remains that the host's tissues undergo biological changes under the influence of antibiotics and then respond to *Candida* infections (6,10-13). Indications of lowered resistance due to the action of aureomycin were seen in our experiments. If the animal's normal defense mechanism against *Candida* was allowed to act for 8-24 hours, the subsequent injection of aureomycin was almost entirely without harm. If the order of injections was reversed, the preceding aureomycin treatment interfered with the defensive reaction of the mouse. Infection 8 or 24 hours later shows still enhanced virulence of *Candida*.

Lowering of resistance against infections has been revealed as the result of mucin action in animals(14,15) and as an effect of cortisone (16,17). Aureomycin may well join this group of biological activators as illustrated in its mode of action against *Candida*.

Summary. 1. *Candida* suspensions are non-pathogenic, and aureomycin solutions non-toxic, when either of these alone is injected intraperitoneally into mice. The mixture of both is fatal. 2. A similar "drug-fungus" effect though less pronounced was observed when aureomycin was given from 24 hours before to 4 hours after *Candida* inoculation. Given 8 and 24 hours after infection aureomycin was ineffective. 3. The virulence enhancing activity of aureomycin was destroyed on standing at room temperature or by boiling, a parallel to its antibiotic activity. 4. No indication of toxin production or biological alteration of *Candida* was found *in vitro*. 5. No direct growth stimulating effect by aureomycin on *Candida* was observed in cultures. The action *in vivo* was one of lowering the animal's resistance.

---

1. Keefer, Ch., *Am. J. Med.*, 1951, v11, 665.

2. Council on Pharmacy and Chemistry, *J.A.M.A.*, 1951, v145, 1276.

3. Kratzer, F. H., Grau, C. R., Starr, M. P., and Reynolds, D. M., pres. at Am. Inst. of Nutrition, May, 1951, in press.

4. Weber, E. M., Luther, H. G., and Reynolds, W. M., pres. at Sympos. Inter. Res. Lab. Microbiol. Rome, 1951.

5. Elam, J. F., Gee, L. L., and Couch, J. R., Proc.

SOC. EXP. BIOL. AND MED., 1951, v78, 832.

6. Woods, J. W., Manning, J. H. Jr., and Patterson, C. N., *J.A.M.A.*, 1951, v145, 207.

7. Moore, M., *J. Lab. and Clin. Med.*, 1951, v37, 703.

8. Campbell, C. C., and Saslaw, S., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 562.

9. Foley, G. E., and Winter, W. D., Jr., *J. Inf. Dis.*, 1949, v85, 268.

10. Harris, H. J., *J.A.M.A.*, 1950, v142, 161.

11. Zimmerman, L. E., *Arch. Pathol.*, 1950, v50, 591.

12. Tomaszewski, T., *Brit. Med. J.*, 1951, v1, 388.

13. Reiches, A. J., *Arch. Dermat. and Syph.*, 1951, v64, 604.

14. Olitzki, L., *Bact. Rev.*, 1948, v12, 149.

15. Strauss, R. E. and Kligman, A. M., *J. Inf. Dis.*, 1951, v88, 151.

16. Antopol, W., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 262.

17. Southam, C. M., and Babcock, V. I., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 105

Received February 13, 1952. P.S.E.B.M., 1952, v79.

Reduced Antibody Titers in Mice Treated with Carcinogenic and Cancer Chemotherapeutic Agents. (19419)

R. A. MALMGREN, B. E. BENNISON,* AND T. W. MCKINLEY, JR.
(Introduced by H. B. Andervont.)
From the National Cancer Institute, Bethesda, Md.†

It has long been known that x-radiation, in addition to inhibiting(1) and initiating(2) neoplastic growth, depresses antibody levels in experimental animals(3). More recently this triad of effects has been demonstrated for nitrogen mustard(4-6). Further it has been reported that the Rous sarcoma virus in addition to initiating tumors will also depress antibody levels(7). Chemotherapeutic substances which have recently been shown to inhibit both tumor growth and antibody production are A-methopterin(8), benzene(9), cortisone (10) and 8-azaguanine(11).

In view of the foregoing observations the following work was undertaken to determine whether or not antibody inhibition is a property common to other substances involved in tumor production and inhibition.

Materials and methods. The data are based chiefly on results obtained in homozygous strain C (B alb C) mice 2 to 6 months old. However, the tests were frequently repeated in homozygous strain C3H mice also 2 to 6 months old, and the same results were obtained. The antibody forming mechanism was evaluated by determining sheep cell hemolysin titers of mouse serum harvested 5 days after the intraperitoneal injection of 0.5 cc of a 4% suspension of washed sheep cells in saline. The hemolysin titers were determined by noting the degree of hemolysis resulting from 30 minute incubation at 37°C of 0.5 cc of serial 2-fold dilutions of test sera with 1.0 cc of 1:20 guinea pig serum and 0.5 cc of 1% sheep cells. Carcinogenic compounds and their non-carcinogenic analogues from three major groups of carcinogens were studied. These included 1) methylcholanthrene, 1,2,5,6-dibenzanthracene, 1,2-benzanthracene‡ and phenanthrene,§ 2) butter yellow (ρ-dimethylaminoazobenzene) and ρ-diethylaminoazobenzene,§ 3) urethan (ethyl carbamate), isopropyl carbamate,‡ β-chloroethylcarbamate,§ and methyl carbamate.§‖ Test sub-

---

* Present address: Division of Chronic Diseases and Tuberculosis, Public Health Service, Federal Security Agency, Washington 25, D.C.

† National Institutes of Health, Federal Security Agency.

‡ An analogue with weak carcinogenic activity.

§ Non-carcinogenic analogue.

‖ We wish to express our thanks to the members of this institute, Dr. J. Hartwell for the alpha peltatin, colchicine, podophyllotoxin, phenanthrene and ρ-diethylaminoazobenzene. Dr. C. D. Larsen for the β-chloroethyl carbamate, isopropyl carbamate and methyl carbamate, Dr. J. White for the ρ-dimethylaminoazobenzene and 1,2,5,6-dibenzanthracene. A-methopterin and triethylene melamine were kindly supplied by Lederle Laboratories Division of

TABLE I. Effect of Carcinogens and Their Non-Carcinogenic Analogues Given Over a Period of 12 Days on Hemolysin Titer of Strain C and C3H Mice.

Test substance	No. of inj.	Dose, mg/kg/inj.	Hemolysis* in anti sera diluted							
			1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Corn oil (control)	3		0	0	0	0	0	0	4+	4+
Methylcholanthrene†	3	25	0	0	0	0	4+	4+	4+	4+
		50	0	0	4+	4+	4+	4+	4+	4+
		100	0	4+	4+	4+	4+	4+	4+	4+
1,2,5,6-dibenzanthracene†	3	50	0	0	0	0	2+	3+	4+	4+
		100	0	0	0	0	3+	4+	4+	4+
		400	0	0	2+	4+	4+	4+	4+	4+
1,2-benzanthrene†	3	100	0	0	0	0	0	3+	4+	4+
		400	0	0	4+	4+	4+	4+	4+	4+
Phenanthrene†	3	800	0	0	0	0	0	0	4+	4+
		1600	0	0	0	0	0	0	4+	4+
Butter yellow (ρ-dimethylaminoazobenzene)†	3	250	0	0	0	0	0	0	4+	4+
		500	0	0	0	2+	3+	4+	4+	4+
		1000	4+	4+	4+	4+	4+	4+	4+	4+
ρ-diethylaminoazobenzene†	3	1000	0	0	0	0	0	2+	4+	4+
		2000	0	0	0	0	0	3+	4+	4+
		4000	0	0	0	0	0	3+	4+	4+
Saline (control)	12		0	0	0	0	0	0	4+	4+
Urethan	12	100	0	0	0	0	0	4+	4+	4+
		200	0	0	0	0	4+	4+	4+	4+
		400	4+	4+	4+	4+	4+	4+	4+	4+
β-chloroethylcarbamate	12	200	0	0	0	0	0	3+	4+	4+
		400	0	0	3+	4+	4+	4+	4+	4+
Isopropyl carbamate	12	800	0	0	0	0	2+	3+	4+	4+
		1000	0	0	0	2+	3+	4+	4+	4+
Methyl carbamate	12	400	0	0	0	0	0	2+	3+	4+
		800	0	0	0	0	0	0	3+	4+

* Zero indicates no turbidity or complete hemolysis and 4+ indicates complete turbidity or no hemolysis; 1+, 2+, 3+ represent intermediate grades of turbidity.

† In corn oil.

stances from the chemotherapeutic group were 1) alpha peltatin, 2) A-methopterin, 3) benzene, 4) colchicine, 5) 2,6-diaminopurine, 6) furacin, 7) podophyllotoxin, 8) sodium arsenite, 9) triethylene melamine, and 10) urethan.¶‖

To show whether or not the effect of the compounds was related to the amount injected, each test substance was administered at several dose levels using 3 to 4 mice for each group. Particularly in the carcinogenic group the maximum tolerated dose was determined for each compound in an attempt to provide a standard by which to compare the toxicities of the carcinogens with their non-carcinogenic analogues. In all experiments control mice were treated with the appropriate vehicle. Test substances were given subcutaneously in a volume of 0.01 cc/g body weight once a day for 5 days starting the day the antigen was administered. Because of the probable slower absorption of the oil soluble carcinogens, these compounds were given in 3 injections spread evenly in a 12-day period which included the 5 days following administration of the antigen. Since urethan is classed as both a carcinogen and a chemotherapeutic agent it was tested on the 5- as well as the 12-day schedule. Because of its rapid rate of breakdown(12) urethan was given daily in all tests. To determine whether all or part of the decreased antibody titers were due to direct effects of the test substances on the antisera, the maximum daily dose of each compound was combined *in vitro* with the antisera before an hemolysin titer was performed. The possibility that the lowered hemolysin titer was due to increased

---

the American Cyanamid Co., Pearl River, N. Y. Furacin was kindly provided by Eaton Laboratories, Norwich, N. Y., and 2,6-diaminopurine by the Wellcome Research Laboratories, Tuckahoe, N. Y.

¶ Also carcinogenic.

TABLE II. Effect of Chemotherapeutic Agents Given Daily Over a Period of 5 Days on Hemolysin Titers in Strains C and C3H Mice.

Test substance	Dose, mg/kg/day	Hemolysis* in antisera diluted								
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Control†		0	0	0	0	0	0	3+	4+	4+
α-peltatin	1.25	0	0	0	0	2+	4+	4+	4+	4+
	2.5	0	4+	4+	4+	4+	4+	4+	4+	4+
	5	4+	4+	4+	4+	4+	4+	4+	4+	4+
A-methopterin	.18	0	0	0	0	3+	4+	4+	4+	4+
	.36	0	0	3+	4+	4+	4+	4+	4+	4+
	.75	0	3+	4+	4+	4+	4+	4+	4+	4+
	1.5	4+	4+	4+	4+	4+	4+	4+	4+	4+
Benzene	3200	0	0	0	0	0	3+	4+	4+	4+
	4800	0	0	0	0	3+	4+	4+	4+	4+
Colchicine	.3	0	0	0	0	0	0	0	3+	4+
	.6	0	0	0	0	2+	4+	4+	4+	4+
	1.2	4+	4+	4+	4+	4+	4+	4+	4+	4+
2,6-diamino-purine	25	0	0	0	0	0	2+	4+	4+	4+
	50	0	0	0	0	2+	4+	4+	4+	4+
	100	0	2+	4+	4+	4+	4+	4+	4+	4+
Furacin	500	0	0	0	0	0	0	0	3+	4+
	1000	0	0	0	0	0	0	0	3+	4+
Podophyllotoxin	1.25	0	0	0	0	4+	4+	4+	4+	4+
	2.5	4+	4+	4+	4+	4+	4+	4+	4+	4+
Sodium arsenite	2.5	0	0	0	0	0	3+	4+	4+	4+
	5	0	0	0	0	3+	4+	4+	4+	4+
	10	0	0	0	3+	4+	4+	4+	4+	4+
Triethylene melamine	.25	0	0	0	2+	3+	4+	4+	4+	4+
	.5	0	3+	4+	4+	4+	4+	4+	4+	4+
Urethan	250	0	0	0	0	0	0	0	0	3+
	500	0	0	0	0	0	3+	4+	4+	4+
	1000	0	0	0	0	3+	4+	4+	4+	4+

* Zero indicates no turbidity or complete hemolysis and 4+ indicates complete turbidity or no hemolysis; 1+, 2+, 3+ represent intermediate grades of turbidity.

† Results obtained with the separate vehicles are indicated by one control group because the titers obtained were the same for all.

destruction of the antibody by the compound *in vivo* was evaluated by administering the test material once a day for 5 days to animals which were injected intraperitoneally on the third day of treatment with 0.5 cc of a mouse anti-sheep red cell serum previously prepared in the same strain of mice. Forty-eight hours later the sera were harvested by decapitation and hemolysin titers performed.

Results and discussion. As shown in Tables I and II, treatment with both the carcinogens and the chemotherapeutic agents, with the exception of furacin, resulted in a significant decrease in hemolysin titers. The degree of inhibition varied from one compound to another but a direct relationship between the amount of any one compound administered and the inhibition it produced was apparent. These results suggest that antibody inhibition may be a property common to substances associated with the initiation and the inhibition of neoplasms. The fact that the non-carcinogenic analogues had little or no effect on the hemolysin titers when administered in quantities several times greater than the corresponding carcinogens brings out a parallelism between the chemical specificity of antibody inhibition and carcinogenic effect.

Although urethan reacted as did all the other substances in the carcinogenic group, its dual role as a carcinogen and a chemotherapeutic agent and the varied biological activity of its analogues makes a more detailed observation interesting. When contrasted with methyl carbamate, which is neither carcinogenic nor chemotherapeutic, the antibody inhibiting ability of urethan and the lack of effect of methyl carbamate follows the pattern

TABLE III. Example* of *in vivo* and *in vitro* Effect of Carcinogenic and Chemotherapeutic Agents on Hemolysin Titers.

Type of test	Sera	Hemolysis† by dilutions of antisera							
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
In vivo	Urethan treated	0	0	2+	4+	4+	4+	4+	4+
	Control	0	0	2+	4+	4+	4+	4+	4+
In vitro	Urethan treated	0	0	0	0	0	0	4+	4+
	Control	0	0	0	0	0	0	4+	4+

* Since none of the compounds tested effected the hemolysin titers *in vivo* or *in vitro* the results obtained with urethan are presented as typical.

† Zero indicates no turbidity or complete hemolysis and 4+ indicates complete turbidity or no hemolysis; 1+, 2+, 3+ representing intermediate grades of turbidity.

of results obtained with other compounds. However, isopropyl carbamate, which has been shown to have some carcinogenic activity(13), and β-chloroethyl carbamate, which interferes with mitosis in a manner and degree similar to urethan(14), both reduced the hemolysin titers although to a lesser degree than the corresponding dose of urethan.

No data are available to explain the inability of furacin to inhibit the hemolysin titer. It might be conjectured that its mechanism of action is different from that of the other compounds or, although it seems unlikely, the ability of all the other agents to reduce hemolysin titers is an unusual coincidence.

The failure of the test substances to inhibit passive antibodies *in vivo* (Table III) suggests that all these compounds act at the site of antibody production. This does not, however, imply that they all achieve their inhibition in the same manner. Table III also indicates that antibodies were not affected *in vitro* by any of the test substances. The observed depression of the antibody titer was, therefore, not a result of interference with the hemolytic test system.

No attempt was made in this work to clarify the way in which these compounds act. However, the question of toxicity was explored partly to establish a standard by which to measure test substances and also to determine whether or not toxicity *per se* was responsible for antibody depression. With this in mind, sodium cyanide, which is not associated with malignancy but which is highly toxic, was given in the maximum tolerated dose of 4 mg/kg body wt/day. At this level sodium cyanide did not lower the hemolysin titer.

A further comparison was made with methylcholanthrene, which was found to have 4 times the lethal effect of its analogue phenanthrene. Four hundred mg/kg body weight given 3 times in a 12-day period was found to be the maximum tolerated dose for methylcholanthrene and 1600 mg/kg body weight given 3 times in a 12-day period the maximum tolerated dose for phenanthrene. As shown in Table I, however, 3 injections of 100 mg/kg body weight methylcholanthrene which is ¼ the toxic dose produced marked antibody inhibition while 3 injections of 1600 mg/kg body weight phenanthrene which equals the maximum tolerated dose of this substance produced no inhibition. On the basis of these findings it would not appear that general systemic toxicity is responsible for the decrease in hemolysin titer.

The marked lowering of the antibody levels by the carcinogens may or may not be related to their carcinogenic activity. However, this reduction of hemolysin titers does suggest that in addition to a very gradual, localized change, thought to be associated with their tumor inducing ability, these compounds also produce a rather sudden systemic alteration which is reflected by the antibody producing system.

Summary. A number of cancer chemotherapeutic agents, carcinogens and their non-carcinogenic analogues were tested for their effect on sheep cell hemolysin production in mice. All of the chemotherapeutic agents, with the exception of furacin, depressed the antibody titers. All of the carcinogens also produced a marked depression of the antibody levels but their non-carcinogenic analogues were without effect. None of the substances

tested inhibited preformed antibodies *in vivo* or *in vitro*.

1. Wood, F. C. and Prime, F., *J. A. M. A.*, 1920, v74, 308.

2. Rowntree, C., *Brit. Med. J.*, 1922, v2, 1111.

3. Hektoen, L., *J. Infect. Dis.*, 1915, v17, 415.

4. Heston, W. E., *J. Nat. Cancer Inst.*, 1950, v11, 415.

5. Phillips, F. S., Hopkins, F. H., and Freeman, M. L. H., *J. Immunol.*, 1947, v55, 289.

6. Rhoads, C. P., *J.A.M.A.*, 1946, v131, 656.

7. Parfentjev, I. A., and Duran-Reynals, F., *Science,* 1951, v113, 690.

8. Oleson, J. J., *Trans. N. Y. Acad. Sc.*, 1950, v12, 118.

9. Hektoen, L., *J. Infect. Dis.*, 1916, v19, 69.

10. Germuth, F. G., and Ottinger, B., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 815.

11. Malmgren, R. A., Bennison, B. E. and McKinley, T. W., Jr., *J. Nat. Cancer Inst.*, in press.

12. Skipper, H. E., Bennett, L. L., Bryan, C. E., White, L., Newton, M. A., and Simpson, L., *Cancer Res.*, 1951, v11, 46.

13. Larsen, C. D., *J. Nat. Cancer Inst.*, 1947, v8, 99.

14. Dustin, P., Jr., *Brit. J. Cancer,* 1947, v1, 48.

Received February 14, 1952. P.S.E.B.M., 1952, v79.

Pancreatic Disease in Mothers of Suckling Mice Infected with Connecticut No. 5 Strain of Coxsackie Virus.* (19420)

LAWRENCE J. KUNZ, SHEILA RICHARDSON, AND ALWIN M. PAPPENHEIMER.

From the Department of Bacteriology and Immunology, Harvard Medical School.

It has been the practice in this laboratory, when infecting suckling mice with one or another strain of Coxsackie virus, to discard the mother at the completion of the experiment. But when it was shown that adult mice are susceptible to infection with the Connecticut No. 5 strain(1), it was decided to keep some of the mothers under observation in order to determine whether or not they might become spontaneously infected.

Thus, the mothers of 19 litters were examined microscopically at various intervals after infection of their young with Conn.-5 virus. Seven of these showed histologic evidence of pancreatic disease, which appears to be the principal pathologic effect of infection of adult mice with the Conn.-5 strain. Microscopic examination of the pancreas and liver of mothers naturally infected through their offspring reveals the same pathologic lesions which are characteristic of pancreatic disease produced experimentally in adult mice(1). Fig. 1 is a photomicrograph of a section of pancreas of a mother mouse which was found dead 9 days after inoculation of her young and 6 days after she had ingested 2 of them. There is a massive early necrosis of the entire pancreas. There was also severe hepatitis, with hyaline necrosis of many liver cells, and fatty infiltration of others, and many phospholipid containing cells in the sinusoids. In general, the character of the pancreatic lesions coincided fairly well with the period elapsing between the presumed ingestion of the infected young and the day of death or sacrifice. Thus there was massive necrosis in those mice surviving only 6 days, whereas those which lived 10 to 12 days presented subacute lesions (Fig. 2). At this time the necrotic acinar cells have been removed and the remaining pancreas consists almost entirely of islands and ducts embedded in loose areolar or fatty tissue thickly infiltrated with histiocytes. Hepatitis of varying degrees of severity was found in some animals. Fig. 3 shows extreme fatty infiltration of the liver, with necrosis of individual liver cells.

The more obvious methods by which the mothers might acquire this viral infection from their offspring are: 1) by ingestion of the excreta of the sucklings, 2) by eating the sick or dead mice in their litters, and 3) by inhalation of infectious materials. In addition to the infected baby mouse carcass, both urine

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

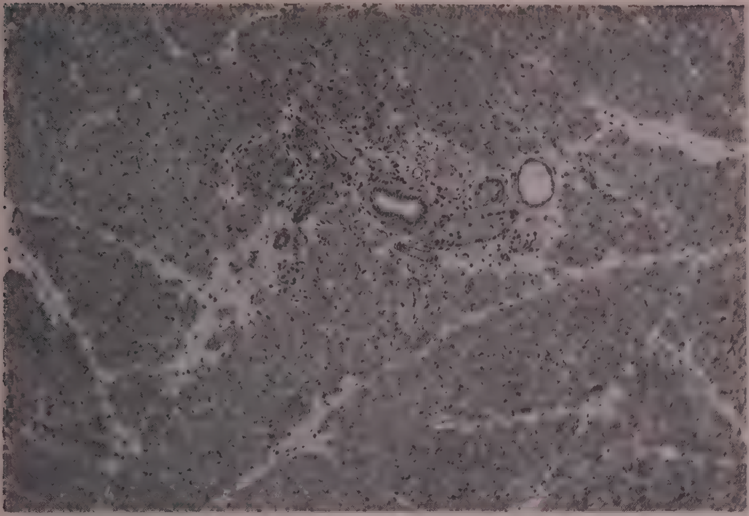


FIG. 1. Mouse 4600. Mother died 9 days after inoculation of offspring with 10^{-3} Conn.-5 virus, 6 days after ingestion of 2 dead young. Massive necrosis of entire pancreas. Island of Langerhans and ducts have escaped destruction. HE × 100.

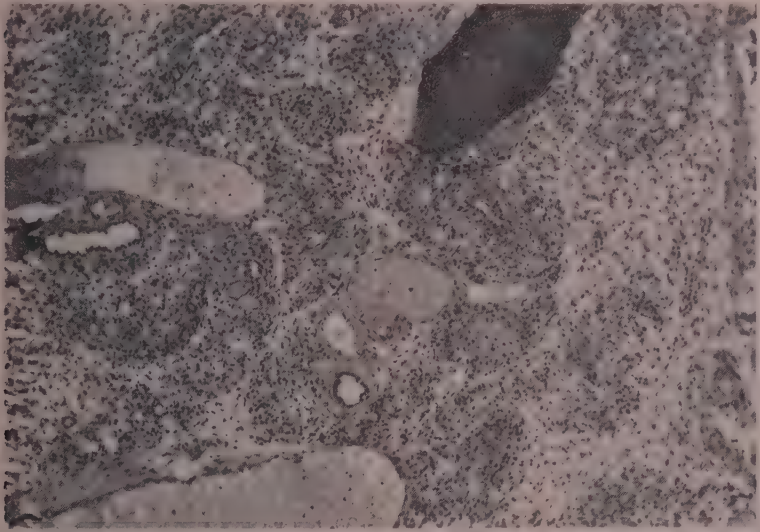


FIG. 2. Mouse 4637. Mouse sacrificed 16 days after inoculation of offspring with 10^{-4} suspension of Conn.-5 virus. Pancreas: Complete loss of exocrine tissue; interstitial histiocytic reaction; preservation of islands and ducts. HE × 83.

(2) and feces(3) contain active virus.

In order to determine the manner in which the mothers most probably become infected, the following experiment was performed: Ten litters (6 to 9 mice per litter) of 0- to 1-day-old mice were inoculated intraperitoneally with approximately 1000 LD_{50} of Conn.-5 virus. The litters, with their mothers, were kept in individual cages for 72 hours. Close attention was paid to the number of sucklings which disappeared during this time—presumably eaten by the mothers. On the 4th day all of the mice were removed from the original cages and redistributed in the following man-

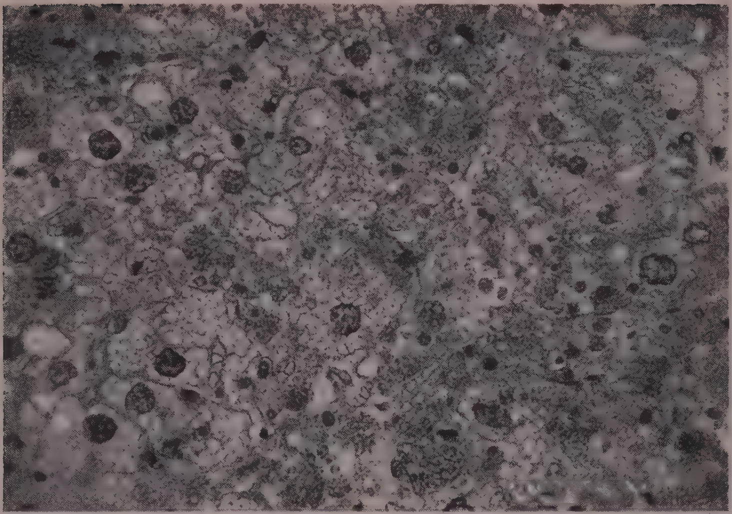


FIG. 3. Mouse 4599. Mother died 17 days after inoculation of offspring with 10^{-2} Conn.-5 virus, 10-12 days after ingesting 2 dead young. Liver: Extreme fat infiltration; necrosis of scattered parenchymal cells. HE × 400.

ner. Each mother was placed in a new individual cage for further observation. Normal 7-week-old mice were exposed to the infected material of the original cages by being quartered in them, one to a cage, until the termination of the experiment. The carcasses of the infected suckling mice were fed to an additional group of normal adults. Ten 7-week-old mice in individual cages were each given 3 sucklings which they rapidly consumed. All of the mice were observed for an additional 15 days, after which they were sacrificed for histologic examination. The results, presented in Table I, indicate that adult mice are probably infected neither by ingesting excreta of their young in the first 3 days after inoculation nor by the cage litter which has been contaminated by the infected suckling mice. Rather, it would appear that adult mice can and do develop pancreatic disease after ingesting the carcasses of infant mice infected with Conn.-5 virus.

TABLE I. Experimental Pancreatic Disease in Adult Mice.

Exp. conditions	No. mice	Sex	Pancreatic disease Present	Absent
1. Mothers of infected litters*	10	♀	0	10
2. Quartered in dirty cages	8	♀	0	8
	2	♂	0	2
3. Consumed infected carcasses	5	♀	3	2
	5	♂	4	1

* Four of the mothers ate one or 2 carcasses during the first 3 days.

In other experiments it was impossible to produce pancreatic disease in adult mice by feeding 10000 LD_{50} of Conn.-5 virus in a dough of whole wheat flour and skim milk; in another experiment one out of 6 adult mice developed pancreatic disease after ingestion of a single carcass of an infected suckling mouse. Moreover, a single attempt to infect adult mice by the intranasal instillation of Conn.-5 virus was unsuccessful as judged by the failure to find signs of pancreatic disease in the inoculated animals.

Evidence that the pancreas of naturally infected mothers contained virus was obtained by inoculating into 0-day-old mice, 10^{-2} and 10^{-4} dilutions of a pool of pancreas tissue suspension obtained from 3 mothers. All of the inoculated mice succumbed and 2 of them, which were examined histologically, showed the usual pancreatitis and hepatitis.

Discussion. There have been few previous studies bearing on the possibility of infection with Coxsackie viruses by oral administration. Melnick and associates(4,5) have found that chimpanzees and cynomologous monkeys, though showing no apparent illness which could be ascribed to the virus, readily became carriers and developed neutralizing antibodies. Kaplan and Melnick(6) have also shown that newborn mice are susceptible to oral infection with 5 different strains (4 immunologic types) of C virus, though not all of the mice became infected. Adult lactating females could not be infected with the High Point strain by feeding. The authors say that they have "observed many adult female mice who cannibalized their infected young without effect on the mothers." Whether this included mothers of sucklings infected with the Conn.-5 strain is not stated.

Since it has been shown that the mothers may become spontaneously infected with the Conn.-5 strain of Coxsackie virus and since those that survive may develop neutralizing antibodies which, by placental transmission or through lactation might protect their offspring, it is obvious that subsequent litters would be unsuitable for experiments with this strain. That this might also apply to other strains of Coxsackie virus is suggested by the recent finding that the Powers(7) and DeMole(8) strains are also capable of producing pancreatitis in adult mice(9).

Summary. Mothers of suckling mice may spontaneously become infected with Conn.-5 strain of Coxsackie virus, following inoculation and infection of their young with this virus. They develop pancreatic lesions and, in some cases, hepatic lesions identical with those produced after intraperitoneal injection of virus. This condition can be reproduced experimentally in adult mice of either sex by feeding them with the carcasses of infected suckling mice.

---

1. Pappenheimer, A. M., Kunz, L. J., and Richardson, S., *J. Exp. Med.*, 1951, v94, 45.
2. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 405.
3. Melnick, J. L., *Bull. N. Y. Acad. Med.*, 1950, v26, 342.
4. Melnick, J. L., Shaw, E. W., and Curnen, E. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 344.
5. Melnick, J. L. and Ledinko, N., *J. Immunol.*, 1950, v64, 101.
6. Kaplan, A. S. and Melnick, J. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 312.
7. Cheever, F. S., Daniels, J. B., and Hersey, E. F., *J. Exp. Med.*, 1950, v92, 153.
8. Weller, T. H., Enders, J. F., Buckingham, M., and Finn, J. J., Jr., *J. Immunol.*, 1950, v65, 337.
9. Unpublished data.

Received February 14, 1952. P.S.E.B.M., 1952, v79.

Influence of Glomerular Filtration Rate upon the Renal Tubular Reabsorption of Sodium.*† (19421)

MATTHEW N. LEVY AND JAY L. ANKENEY.‡ (Introduced by Ewald E. Selkurt.)
From the Department of Physiology, Western Reserve University School of Medicine, Cleveland, O.

In a recent study(1), the administration of hypertonic saline to dogs resulted in an increased rate of sodium reabsorption in every case. Since the rise in plasma sodium concentration was accompanied by a variable, but consistently present, elevation of glomerular filtration rate in each experiment, it was not possible to determine the factor which was responsible for the altered rate of sodium reabsorption. Other investigators also have observed changes in glomerular filtration rate subsequent to hypertonic saline infusions, the usual effect being an augmentation(2-3), although reduced filtration rates have occurred in some cases(4).

In the present series of experiments the administration of hypertonic saline exerted only a negligible effect upon the rate of glomerular filtration in six out of eight experi-

---

[*] This paper was presented in abridged form at meeting of the American Physiological Society, April, 1951.

[†] Supported by a grant from the U. S. Public Health Service.

[‡] U. S. Public Health Service Postgraduate Research Fellow.

ments. This made it possible to distinguish the acute effects of elevated plasma sodium levels from those due to changes in filtration rate upon the rate of renal tubular reabsorption of sodium.

Methods. Eight acute experiments were performed upon male dogs anesthetized with sodium pentobarbital, 30 mg/kg. Urine was collected by means of plastic catheters inserted directly into the ureters through a suprapubic incision. After a pair of control clearance periods the plasma sodium level was progressively elevated by means of a continuous intravenous infusion of 3% saline. Eight successive clearance periods were then obtained beginning soon after the hypertonic saline infusion had been started. The rate of saline administration was gradually augmented after every 2 periods. Plasma sodium levels were elevated for a total of only one hour or less in each experiment. Glucose Tm was determined in 5 of these experiments, and PAH Tm in the other 3, in an attempt to evaluate the effects of hypertonic saline upon renal tubular function. The clearance of creatinine was used to measure the glomerular filtration rate in all experiments. Creatinine was determined in plasma filtrates and urine by the method of Folin and Wu(5); PAH by the method of Smith *et al.*(6); and glucose by the method of Benedict(7). The sodium content of all urine and plasma samples was determined with a Perkin-Elmer flame photometer employing an internal lithium standard. No corrections were made in our calculations for the Donnan effect.

Results. Fig. 1 illustrates the changes which occurred in glomerular filtration rate (C_{Cr}), the rate of sodium reabsorption (T_{Na}), and the maximum secretory capacity for PAH (Tm_{PAH}) in 3 experiments following the administration of hypertonic saline. It is evident from the figure that only slight changes in C_{Cr} and T_{Na} are manifest in 2 of these experiments, represented by the squares and triangles. In both of these experiments, C_{Cr} remained within ± 4 cc/min of the mean value for that experiment, and T_{Na} did not vary by more than ± 0.6 mEq/min from the mean rate of sodium reabsorption, despite wide variations in the plasma sodium concentration (P_{Na}). This relatively constant rate of sodium reabsorption in the face of significant increases in sodium load resulted in tremendous increases in the rate of sodium excretion.

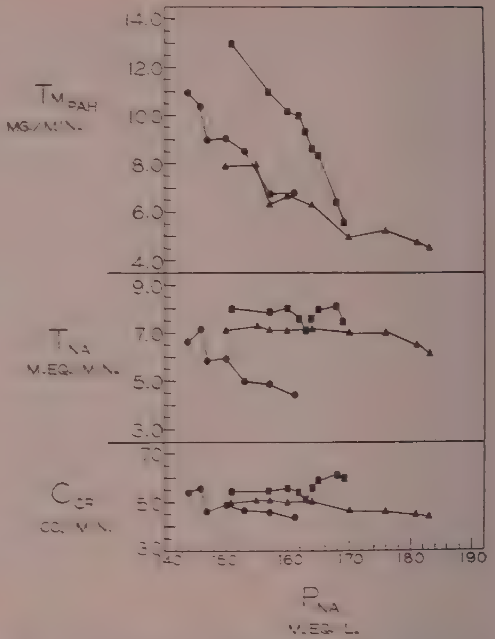


FIG. 1. Effects of hypertonic saline upon PAH Tm, sodium reabsorption (T_{Na}), and glomerular filtration rate (C_{Cr}).

In the third experiment, represented by the circles in Fig. 1, C_{Cr} dropped from a value of 54 cc/min at a P_{Na} of 144 mEq/1 to 44 cc/min when P_{Na} was elevated to 161 mEq/1. Under these conditions the T_{Na} fell from 6.7 to 4.4 mEq/min, a decrease which is relatively greater than the decline in C_{Cr}. In all 3 animals the elevation of P_{Na} obviously exerted a marked depressant effect upon the Tm_{PAH}. The Tm values at elevated plasma sodium levels were 43, 58, and 62% of control.

Five similar experiments were performed in which the glucose Tm (Tm_G) was determined instead of the PAH Tm, and these experiments are illustrated in Fig. 2. In this figure, the greatest alteration in glomerular filtration rate is manifest in the experiment represented by the open circles. There was a rise in this experiment from 54 cc/min at a P_{Na} of 144 mEq/1 to a rate of 73 cc/min

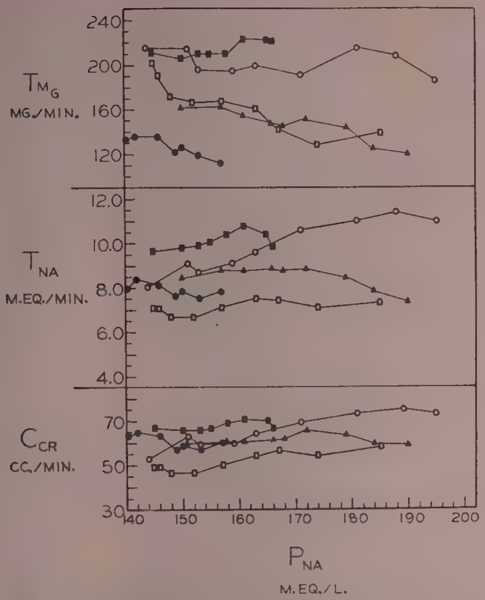


FIG. 2. Effects of hypertonic saline upon glucose Tm, sodium reabsorption, and glomerular filtration rate.

when the P_{Na} was 195 mEq/1. Under these conditions the rate of sodium reabsorption increased from 8.0 to 10.9 mEq/min, tending to parallel the change in C_{Cr}. The results of this experiment closely simulate those of the previous studies reported by Selkurt and Post(1).

In the other 4 experiments included in Fig. 2, the C_{Cr} changed to a much lesser extent, and the T_{Na} also remained relatively constant over a wide range of plasma sodium concentrations. In these 4 experiments the greatest change in C_{Cr} was ± 7 cc/min from the mean value for that experiment (open squares), and the variation was significantly less in the remaining experiments of this group. The greatest deviation in T_{Na} was ± 0.75 mEq/min from the mean (solid triangles). In all experiments the rate of sodium excretion increased markedly as P_{Na} rose.

When Fig. 2 is compared to the preceding figure, it is obvious that Tm_{PAH} was much more severely depressed than was Tm_G for equivalent increases in P_{Na}. The glucose Tm varied from 32% below control to 5% above control following the administration of hypertonic saline, with an average depression to 16% below control for the 5 experiments.

Discussion. When the plasma sodium concentration was progressively elevated without incurring any significant alterations of glomerular filtration rate, the rate of sodium reabsorption remained remarkably constant, and the excess sodium was eliminated in the urine. In those experiments in which an increase or decrease in filtration rate did occur, T_{Na} changed concordantly. In the studies of Selkurt and Post(1), C_{Cr} and T_{Na} increased in every case, but the greatest rises in T_{Na} were observed in those experiments where C_{Cr} was augmented the most. Thus, it appears that the rate of sodium reabsorption is independent of acute changes in plasma sodium concentration, but is very sensitive to changes in filtration rate. In these respects the mechanism of sodium reabsorption appears to be similar to that for bicarbonate(8) and chloride (9).

The depressant effect of elevated P_{Na} levels upon the glucose and PAH transport mechanisms is undoubtedly nonspecific in nature. Hypertonic saline has a similar action upon the rates of reabsorption of bicarbonate(8), sulfate(10), and ascorbic acid(11). It was shown by Selkurt and Houck(11) that the effect of hypertonic saline upon the ascorbic acid Tm was not due to the resultant diuresis, for equivalent increases in urine flow produced by mannitol did not affect the rate of ascorbic acid reabsorption. Contrary to our demonstration of the marked depression of Tm_{PAH} by hypertonic saline, Stamler(12) demonstrated that saturation of the tubular secretory mechanism for PAH does not alter the rate of sodium transport. Therefore the depressant effect of elevated P_{Na} upon the Tm_{PAH} is not a true competitive phenomenon, and its exact nature remains obscure. It is evident, however, that neither Tm_{PAH} nor Tm_G should be used to assay effective tubular mass under conditions where significant alterations in P_{Na} are produced.

This very appreciable effect upon the transport mechanisms for glucose and PAH leads us to suspect that tubular function in general might be depressed by hypertonic saline. With respect to the renal tubular reabsorption of sodium in particular, two alternative possibilities should be considered. First, it may be assumed that all aspects of tubular function actually are impaired. In this event the renal tubular cells might tend to reabsorb additional quantities of sodium as the load is increased, but would be prevented from doing so because of the impairment of function. The resultant effect would be the constant T_{Na} which was observed. The second possibility is that only certain discrete enzyme systems are inhibited and that the sodium reabsorptive mechanism is spared, thereby allowing T_{Na} to remain constant, while Tm_{PAH} or Tm_G are significantly reduced. Credence is lent to this possibility by the fact that Tm_{PAH} appears to be much more markedly affected than is Tm_G, indicating that the various transport systems are not all altered to the same extent. At present, we cannot say which of these alternatives is the correct one.

Summary. 1. When the plasma sodium concentration is progressively elevated in dogs by the administration of hypertonic saline, the rate of renal tubular reabsorption of sodium remains constant in those experiments where the glomerular filtration rate is not significantly affected. If the filtration rate increases or decreases in response to the hypertonic saline, the rate of sodium transport deviates in the same direction. Therefore, in experiments where the sodium level is elevated for a period of one hour or less, any alteration in the rate of sodium transport is dependent upon the concomitant change in filtration rate. 2. Hypertonic saline exerts a profoundly depressant effect upon the PAH Tm, and a significant but less marked influence upon the glucose Tm. This depression is believed to be nonspecific in nature and contra-indicates the use of these determinations as an index of effective tubular mass in experiments in which variations in sodium concentration are involved.

---

1. Selkurt, E. E., and Post, R. S., *Am. J. Physiol.*, 1950, v162, 639.
2. Baldwin, D., Kahana, E. M., and Clarke, R. W., *Am. J. Physiol.*, 1950, v162, 655.
3. Wiggins, W. S., Manry, C. H., Lyons, R. H., and Pitts, R. F., *Circulation*, 1951, v3, 275.
4. Green, D. M., and Farah, A., *Am. J. Physiol.*, 1949, v158, 444.
5. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, v38, 81.
6. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, v24, 388.
7. Benedict, S. R., *J. Biol. Chem.*, 1928, v76, 457.
8. Pitts, R. F., and Lotspeich, W. D., *Am. J. Physiol.*, 1946, v147, 138.
9. Lotspeich, W. D., Swan, R. C., and Pitts, R. F., *Am. J. Physiol.*, 1947, v148, 445.
10. Lotspeich, W. D., *Am. J. Physiol.*, 1947, v151, 311.
11. Selkurt, E. E., and Houck, C. R., *Am. J. Physiol.*, 1944, v141, 423.
12. Stamler, J., *Am. J. Physiol.*, 1951, v165, 109.

Received February 14, 1952. P.S.E.B.M., 1952, v79.

A Protective Role of Pyridoxin Against the Toxic Effects of P^{32}.* (19422)

CAMILLO ARTOM, W. E. CORNATZER,† AND GEORGE T. HARRELL, JR.

From the Departments of Biochemistry and Internal Medicine, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N. C.

In a continuation of our study on the effects of internal radiation by P^{32} as related to various dietary factors(1,2), the role of pyridoxin has now been investigated. After

---

* Technical assistance was given by J. Giles and C. Downs. This work was performed under a contract between U. S. Atomic Energy Commission and the Bowman Gray School of Medicine. The P^{32} was obtained from the Oak Ridge National Laboratories. Desoxypyridoxin was graciously supplied by Merck and Co., Rahway, N. J. A preliminary report was presented before the Southeastern Section of the Soc. EXP. BIOL. AND MED., Atlanta, Ga., Jan., 1952.

† Present address, Department of Biochemistry, University of N. Dakota, Grand Forks, N. D.

whole body irradiation of mice with midlethal doses of X-rays, large amounts of pyridoxin prolonged the survival, but did not improve the anemia and leukopenia(3). In the present experiments when pyridoxin was added to a pyridoxin deficient diet with a low protein content, no change in the survival of mice injected with P^{32} was detectable. On the other hand, a marked degree of protection by the vitamin became apparent when the deficiency was made more severe by the administration of a pyridoxin analog (desoxypyridoxin)(4), or by diets which presumably increase the requirements for pyridoxin (high protein diets(5) with added cystine or methionine(6)).

Experimental. Two basal diets were employed, one with a 5% casein content (Diet 34(1)), the other with a higher protein level (Diet 41, containing: Casein 30 p.p. 100, dextrin 29.5, sucrose 29.5, Crisco 4, cod liver oil 1, Ruffex 2, salt mixture (U.S.P. No. 2) 4). A mixture of B vitamins(2) from which pyridoxin had been omitted, was incorporated in the diets. Six series of experiments were made on a total of 558 male albino mice, placed under experimental conditions which have been described(1,2). In each series of experiments the animals were distributed in 4 groups: *a*, *b*, *c*, and *d*. All 4 groups received the same diet and the same supplements, except that pyridoxin HCl (1 mg/100 g of diet unless otherwise indicated) was added to the diet of groups *b* and *d* only. After 3 to 10 days on the experimental diet, the mice of groups *a* and *b* were injected intraperitoneally with a solution of radioactive $Na_2HP^{32}O_4$ (5 or 6 μc/g) and kept under observation for 42 more days. Groups *c* and *d* were used as controls and observed for the same length of time. The criteria previously used as an indication of the toxic effects of the isotope(1,2) were applied also to the results of the present experiments, except that in computing the average length of survival, a survival of 42 days was ascribed to the mice still alive at the end of the experiment.

TABLE I. Survival of Mice Injected with P^{32} (6 μc/g) and Maintained on a Low Protein Diet (Diet 34).*

Groups of mice		Pyridoxin in diet	No. of mice	Survivors 21st day, %	Avg length of survival, days†
I	a	—	24	67	32 ± 2.8
	b	+	15	67	30 ± 2.5
	c‡	—	12	100	42
	d‡	+	12	100	42

* Time of 50% deaths was >42 days for all 4 groups.

† Values preceded by ± are the stand. errors of the means.

‡ Control groups not injected with P^{32}.

TABLE II. Survival of Mice Injected with P^{32} (5 μc/g) and Maintained on a Low Protein Diet (Diet 34) with Added Desoxypyridoxin.

Groups of mice		Pyridoxin in diet*	No. of mice	Time of 50% deaths, days	Survivors 21st day, %	Avg length of survival,† days
II§	a	—	46	13	15	16 ± 1.4
	b	+	46	>42	74	34 ± 1.9
	c‡	—	39	>42	87	38 ± 1.5
	d‡	+	13	>42	100	42
III‖	a	—	30	14	20	20 ± 2
	b	+	27	>42	59	30 ± 1.8
	c‡	—	26	41	93	36 ± 1.5
VI¶	a	—	18	15	22	22 ± 2.6
	b	+	18	>42	78	36 ± 2.5
	c‡	—	24	>42	92	39 ± 1.4
	d‡	+	12	>42	100	42

* 2 mg/100 g of diet, then 1 mg/100 g when desoxypyridoxin was omitted from diet.

† Values preceded by ± are the stand. errors of the means.

‡ Control groups not inj. with P^{32}.

§ Desoxypyridoxin (5 mg/100 g diet) for 9 days.

‖ 7

¶ 5

Results. In the experiments of Series I (Table I) supplementation of the low protein diet with pyridoxin did not alter the survival of the mice injected with P^{32}. The controls maintained on the pyridoxin-deficient diet and not injected with the isotope grew fairly well and did not show skin lesions, thus confirming previous indications(7) that, unlike the rat, the mouse on a moderately low protein diet does not develop typical symptoms of pyridoxin deficiency. In the experiments of Series II, III and IV (Table II) in which the low protein diet contained desoxypyridoxin, survival figures were higher in the groups injected with P^{32} and receiving pyridoxin, in addition to the anti-vitamin. Similar differences between the groups receiving or not re-

TABLE III. Survival of Mice Injected with P^{32} (5 μc/g) and Maintained on a High Protein Diet (Diet 41) with Added Cystine (Series V) or Methionine (Series VI).

Groups of mice		Pyridoxin in diet	No. of mice	Time of 50% deaths, days	Survivors 21st day, %	Avg length of survival,* days
V‡	a	—	23	18	22	21 ± 2.6
	b	+	23	>42	83	36 ± 2.4
	c†	—	24	41	96	37 ± 1.4
	d†	+	19	>42	95	39 ± 1.3
VI§	a	—	28	15	28	21 ± 2
	b	+	27	28	67	27 ± 2.4
	c†	—	24	>42	100	39 ± 1
	d†	+	23	>42	91	40 ± 1

* Values preceded by ± are the stand. errors of the means.

† Control groups not inj. with P^{32}.

‡ Cystine (.3 g/100 g diet) for 52 days.

§ Methionine (.5 g/100 g diet) for 47 days.

TABLE IV. Statistical Appraisal of the Protection by Pyridoxin Against P^{32} Toxicity.

		10% casein + desoxypyridoxin	30% casein + S-containing amino acids
No. of mice inj. with P^{32}		185	101
No. of survivors 21st day	*Chi*^{2}	41	24
	P*	< .01	< .01
Avg length of survival	Diff. between adjusted means	11±1.12†	10±.98†
	t	9.9	10.2
	P*	< .01	< .01

* Probability for a chance occurrence.

† Stand. errors of the diff. between adjusted means.

ceiving pyridoxin are apparent (Table III) in the experiments with a high protein diet supplemented with cystine (Series V), or methionine (Series VI). In the pyridoxin dedeficient groups of all 5 Series of experiments acrodynia appeared early and often progressed to the almost complete loss of hair. Growth of these mice was also definitely impaired. For a statistical evaluation, the results of the experiments of Series II-IV, or Series V and VI, respectively, have been grouped together. The average values from the groups *a* and *b* were then adjusted on the basis of the data from the corresponding controls which did not receive the isotope (groups *c* and *d*). The significance of the differences between these adjusted means was then estimated, using the *Chi*^{2} method for the number of survivors at the 21st day and the *t* test of significance for the average length of survival (Table IV).

Discussion. It is apparent that the administration of pyridoxin to mice which are actually deficient in pyridoxin exerts a significant protection against the injurious effect of P^{32}. This protection is of about the same degree as that observed previously with the combined administration of vitamin B_{12} and folic acid to mice, placed on sulfasuxidine-containing diets and injected with P^{32}(2). It is possible that both groups of findings may be due primarily to a beneficial effect on the recovery of the bone marrow, although evidence for a specific role of pyridoxin in erythropoiesis is somewhat equivocal and restricted to certain animal species (see, for instance(8)). On the other hand, atrophy of the thymus and lymphoid tissue accompanied by changes in the white blood cells is a striking and constant finding in acute pyridoxin deficiency(9). It is tempting to compare our present observations with others(10), suggesting that the integrity of large sections of the lymphatic system (as by lead-shielding the spleen during exposure to X radiation) increases the survival of the animals and hastens the regeneration of hemopoietic tissues.

Summary. Mice placed on pyridoxin-free diets were injected with a single dose of radioactive phosphate (5-6 μc/g). In the animals maintained on a low protein diet (in which symptoms of vitamin deficiency did not appear), the administration of pyridoxin did not affect the survival. On the contrary a marked protection by pyridoxin against the injurious effects of P^{32} was observed in experiments in which the vitamin deficiency had been made more severe by adding a pyridoxin analog (desoxypyridoxin) to the low protein diet, or by increasing the requirements for the vitamin (high protein diets with added cystine or methionine). In these experiments the differences between the groups receiving and those

not receiving pyridoxin were larger than the differences which might result from a simple additive effect of the internal radiation and vitamin deficiency.

---

1. Cornatzer, W. E., Harrell, G. T., Jr., Cayer, D., and Artom, C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 492.

2. Cornatzer, W. E., Artom, C., Harrell, G. T., Jr., and Cayer, D., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 552.

3. Goldfeder, A., Cohen, L., Miller, C., and Singer, M., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 272.

4. Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 125; Porter C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, 1947, v167, 573.

5. Sakurai, Y., and Aoyagi, V., *Bull. Inst. Phys. Chem. Res.* (Tokyo), 1942, v21, 1092; *Chem. Abst.*, 1947, v41, 5934; Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, 1945, v157, 551.

6. Cerecedo, L. R., and Foy, J. R., *Arch. Biochem.*, 1944, v5, 207; Cerecedo, L. R., Foy, J. R., and DeRenzo, E. C., *Arch. Biochem.*, 1948, v17, 397.

7. Foy, J. R., and Cerecedo, L. R., *J. Nutrition*, 1941, v22, 439.

8. Lepkowsky, S., in *Symposium on the Biological Actions of Vitamins*, University of Chicago Press, Chicago, Ill., 1942,

9. Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 90; Mushett, C. W., Stebbins, R. B., and Barton, M. N., *Trans. N. Y. Acad. Sci.*, 1947, v9, 291; Weir, D. R., Heinle, R. W., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 457; Stoerk, H. C., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1302.

10. Jacobson, L. O., Simmons, E. L., Bethard, W. F., Marks, E. K., and Robson, M. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 455; Jacobson, L. O., Simmons, E. L., Marks, E. K., Robson, M. J., Bethard, W. F., and Gaston, E. O., *J. Lab. and Clin. Med.*, 1950, v35, 746.

Received February 18, 1952. P.S.E.B.M., 1952, v79.

Effects of Some Metabolic Analogs on Growth of Mumps and Influenza Viruses in Tissue Cultures.* (19423)

RICHARD T. CUSHING† AND HERBERT R. MORGAN.

From the Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The inhibition of viral multiplication by various metabolic antagonists in tissue cultures has been studied by several investigators, using a number of viruses(1-4). Indeed, this technic is a promising avenue of approach for studying the growth requirements of many viruses. Using this method, certain vitamin, amino acid, and purine analogs were investigated to determine if they would inhibit effectively multiplication of mumps and influenza viruses in cultures of chick embryonic tissue without serious toxic effects on the host tissue.

Methods. Cultures of 9- to 11-day-old chick embryonic tissue were prepared, employing a method similar to that of Earle(5). Perforated cellophane discs cut to size were placed in the bottoms of 10 ml Erlenmeyer flasks which were then autoclaved. Using whole embryo tissue for the cultures to be inoculated with influenza virus, and amniotic membrane tissue for those cultures to be infected with mumps virus, the tissues were minced with scissors in Hanks's(6) balanced salt solution without added sodium bicarbonate. The tissue fragments were then planted in the flasks underneath the cellophane discs. Two ml of nutrient solution composed of 2 volumes of Hanks's solution with sodium bicarbonate and one volume of ox serum ultrafiltrate, to which the virus was added, were then placed into each flask. The viruses employed were strains of mumps and influenza (PR8) viruses which had been cultivated in the allantoic sacs of embryonated eggs. Incubation of the tissue cultures was at 36°C. After 24 hours the infected nutrient

---

* This investigation was aided by a grant from the National Institutes of Health, U. S. Public Health Service.

† Special Student Fellow of the National Foundation for Infantile Paralysis.

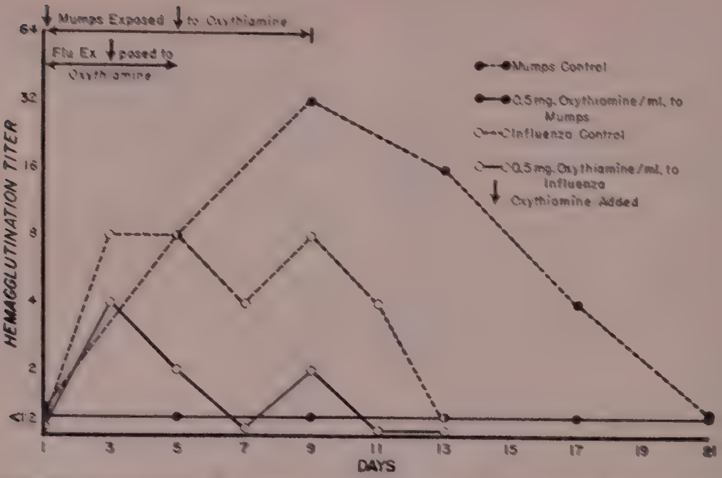


FIG. 1. Inhibition of growth of mumps and influenza (PRS) viruses in tissue culture by oxythiamine.

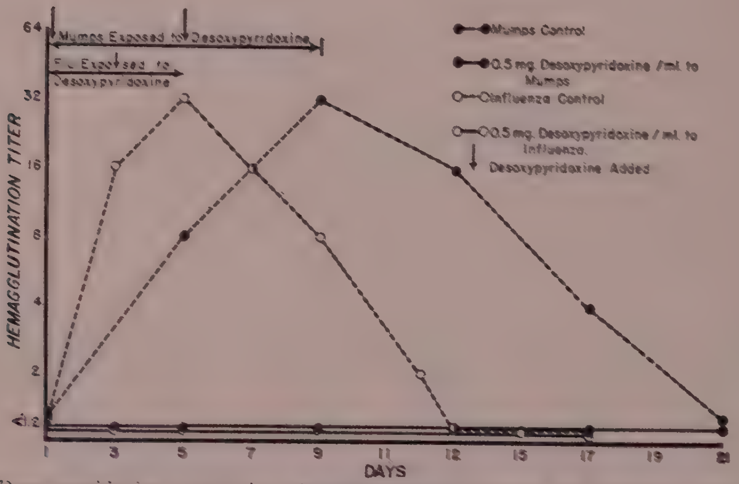


FIG. 2. Desoxypyridoxine suppression of multiplication of mumps and influenza (PRS) viruses in tissue cultures.

fluid was withdrawn and replaced with 2 ml of fresh nutrient solution containing one of the analogs. The analog was added to the cultures for 2 successive fluid changes, after which they were maintained on normal nutrient solution. Fluid changes were made every 48 hours on the influenza cultures, every 96 hours on the mumps cultures, and the fluid removed was tested promptly for virus content by hemagglutination titrations with hen erythrocytes. Duplicate flasks were prepared for each concentration of each analog, and appropriate control cultures were maintained for the duration of each experiment. The experiments were continued until the control cultures showed greatly diminished virus yield. Approximately 100 units of penicillin and 100 μg of streptomycin ml were added to the nutrient fluid to protect against bacterial contamination.

To study the possible toxic effects of the test analogs on chick embryo tissue, fragments of heart tissue from 12- to 14-day-old embryos were planted in plasma clots in Carrel

TABLE I. Effect of Various Analogs on Growth of Mumps and Influenza (PR8) Viruses in Tissue Cultures.

Analog	Concentration, mg/ml	Metabolite	Virus inhibition	
			Flu	Mumps
Desoxypyridoxine	.1	Pyridoxine	0	0
	.5		+	+
Oxythiamine	.1	Thiamine	0	+
	.5		+	+
Pyridine-3-sulfonic acid	.5	Nicotinic acid	0	0
2-Amino-4-hydroxy-6-formylpterine	.2	Xanthine	0	0
Salicyl-β-alanide	.5	Pantothenic acid	0	0
2,6-Diaminopurine	.1	Adenine	0	0
8-Azaguanine	.5	Guanine	0	0
β-2-thienylalanine	.5	Phenylalanine	0	0

flasks. Nutrient fluids containing the analogs were added, and these cultures were incubated for 4 days at 36°C. Daily examinations were made to observe the ability of the fragments to pulsate and to note the outgrowth of fibroblasts at the periphery of the explants.

Results. Two of the vitamin analogs, oxythiamine and desoxypyridoxine, demonstrated a definite inhibitory effect on the multiplication of both mumps and influenza (PR8) viruses, as shown in the growth curves (Fig. 1 and 2). Of the two viruses, mumps appears to be the most sensitive to oxythiamine. Mumps virus growth was markedly inhibited, within the limits of hemagglutination measurement, when exposed to 0.1 mg/ml of oxythiamine, while influenza virus multiplication was only moderately inhibited at 0.5 mg/ml of this analog. Both viruses were markedly inhibited by desoxypyridoxine at a concentration of 0.5 mg/ml but not at 0.1 mg/ml. In the toxicity tests these two compounds, at the concentrations employed, did not produce obvious toxic effects on the chick embryonic heart tissue as measured by contractility and fibroblastic proliferation.

The remaining analogs, pyridine-3-sulfonic acid, salicyl-β-alanide, 2-amino-4-hydroxy-6-formylpterine, 2,6-diaminopurine, 8-azaguanine, and β-2-thienylalanine, produced no significant inhibitory effect on the growth of either virus at the concentrations used (Table I).

Discussion. Since sufficient time (24 hr) for the inoculated virus to invade the host tissue elapsed before the analogs were introduced, it would seem that the effects of these analogs were exerted on intracellular virus rather than by blocking the attachment of the virus to the cells.

Ackermann(7) has presented evidence that the functioning of the Krebs cycle of carbohydrate metabolism is essential for the multiplication of influenza (PR8) virus in tissue cultures. It may be that oxythiamine blocks pyruvate oxidation and entry of pyruvate into the Krebs cycle of the intermediary metabolism of the tissue cells sufficiently to inhibit multiplication of the virus. Using cell preparations containing tyrosine decarboxylase as their test system, Umbreit and Waddell(8) have concluded that desoxypyridoxine exerts its antagonistic effect after being phosphorylated by competing with pyridoxal phosphate for the apoenzyme. It might be postulated that phases of amino acid metabolism, such as transamination in which pyridoxine functions as a coenzyme, are essential to viral multiplication.

The inhibitory effect of oxythiamine and desoxypyridoxine on mumps and influenza (PR8) virus growth was not reversed significantly by the withdrawal of these analogs, at least as measured by the hemagglutination technic. It should be noted, however, that the rapid growth phase of the viruses in the control cultures generally had passed its peak by the time the analogs were removed from the test cultures. Hence, it is not clearly demonstrated that the inhibition was irreversible.

Summary. The vitamin analogs oxythiamine and desoxypyridoxine inhibit the multiplication of mumps and influenza (PR8) viruses in tissue cultures at concentrations shown to be without observable toxic effects on the tissue itself. Other analogs tested, including pyridine-3-sulfonic acid, salicyl-β-alanide, 2-amino-4-hydroxy-6-formylpterine, 2,6-diaminopurine, 8-azaguanine, and β-2-thienylalanine, demonstrated no significant inhibitory effects on the growth of these viruses under the conditions of these experiments.

1. Thompson, R. L., *J. Immunol.*, 1947, v55, 345.
2. Morgan, H. R., Abstracts of Papers, 49th General Meeting, Soc. Am. Bacteriol., Cincinnati, May 1949, p82.
3. Morgan, H. R., *J. Exp. Med.*, in press.
4. Eaton, M. D., Magasanik, B., Perry, M. E., and Karibian, D., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 505.
5. Evans, V. J., and Earle, W. R., *J. Nat. Cancer Inst.*, 1947, v8, 103.
6. Hanks, J. H., and Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.
7. Ackermann, W. W., *J. Biol. Chem.*, 1951, v189, 421.
8. Umbreit, W. W., and Waddell, J. W., PROC. SOC., EXP. BIOL. AND MED., 1949, v70, 293.

Received February 20, 1952. P.S.E.B.M., 1952, v79.

Effect of "Benemid" and Carinamide on Penicillin Therapy of Experimental Pneumococcic Infections in Mice. (19424)

W. F. VERWEY, A. KATHRINE MILLER, AND DOROTHY WILMER SCHLOTTMAN.*
(Introduced by L. E. Arnow.)

From the Department of Bacteriology, Research Division, Sharp & Dohme, West Point, Pa.

Carinamide (4′-carboxyphenylmethanesulfonanilide) is a compound capable of inhibiting the excretion of penicillin by the renal tubules(1). Therefore, higher and more prolonged plasma concentrations of penicillin are produced when carinamide is administered during penicillin therapy(2). The oral administration of carinamide was found to enhance the therapeutic effectiveness of penicillin administered intramuscularly 6 to 16-fold in the treatment of experimental pneumococcic infections in mice(3). The enhanced chemotherapeutic effect demonstrated in these experiments has been confirmed clinically(4), but relatively large doses of carinamide were required in order to maintain the blood level of this compound necessary to suppress the tubular excretion of penicillin.

In the continuing search for compounds capable of suppressing more efficiently the tubular excretion of penicillin, 'Benemid',† [p(di-n propylsulfamyl) benzoic acid], was found to be effective in dogs, was well absorbed when given by mouth, and was excreted so slowly that its renal clearance could not be estimated(5). These results suggested the comparative evaluation of carinamide and 'Benemid' for the inhibition of penicillin excretion using dogs, and the results have been reported(5). In addition to these strictly pharmacologic studies, it was considered advisable to compare these two compounds quantitatively in terms of their effects upon the actual chemotherapeutic activity of penicillin in the treatment of an experimental infection in mice. In the experiments to be described, carinamide and 'Benemid' have been compared with respect to their penicillin-enhancing effects and with respect to the time during which the two compounds are able to increase the therapeutic effect of penicillin through the suppression of its tubular excretion. In addition, determinations were made of the blood level curves resulting from the selected doses of carinamide and 'Benemid' so that the potentiating actions of these two

---

[*] Present address: U. of Washington, Pullman, Wash.

[†] 'Benemid' is the trademark applied to p-(di-n propylsulfamyl) benzoic acid by Sharp and Dohme. The tentative nonproprietary name of this compound is probenecid.

drugs could be related to their plasma concentrations.

Experimental. Female mice[‡] weighing 16-22 g were used. For the chemotherapeutic experiments, mice were infected intraperitoneally with approximately 1000 minimum lethal doses of a Type I pneumococcus contained in 0.5 ml of a diluted 6-hour broth culture. Intramuscular injections of varying concentrations of aqueous sodium penicillin were given in 0.05 ml quantities at the time of infection, designated as zero hour, and again 12 hours after infection. Five or 10 mg of 'Benemid' or carinamide, suspended in 0.5 ml of 0.5% gum tragacanth, were given by stomach tube at zero hour or at 1,2,4,6,8 or 10 hours before each penicillin injection. Infected control mice receiving penicillin but no adjunct therapy were fed 0.5 ml of the tragacanth suspending material at zero hour. Each treatment group consisted of 10 mice and the experiments were terminated 7 days after infection. The dose of penicillin protecting 50% of the mice (PD_{50}) then was calculated for each treatment schedule, using the method of Reed and Muench(6).

Results. It can be seen that the variable in these experiments is the time between the feeding of carinamide or 'Benemid' and the injection of penicillin; therefore, the greater the persistence of the adjuncts in the blood, the greater and more prolonged would be their effect in reducing the penicillin PD_{50}. The results of the experiments have been expressed as the percentage reduction in the dose of penicillin protecting 50% of the animals (PD_{50}) compared to the control values obtained when no adjunct therapy was administered. The data from several separate experiments have been averaged and the effect of the adjunct pre-feeding time on the penicillin PD_{50} is shown in Table I. It will be seen that when either carinamide or 'Benemid' is given orally at the time of penicillin therapy (zero hour), there is a 90% to 97% reduction in the amount of penicillin required to protect 50% of the treated animals. Further experiments have indicated that this magnitude of decrease represents the maximal enhancement of therapy that can be expected from the complete suppression of renal tubular excretion of penicillin. Absorption of the two adjuncts from the intestinal tract apparently is rapid since no increase in effect is noted when the compounds are fed one hour before the injection of penicillin. These figures also demonstrate that the 5 mg dose of carinamide maintained maximal effectiveness for one hour, but that a 5 mg dose of 'Benemid' maintained the same maximal effectiveness for about two hours. Correspondingly, when the 10 mg doses of these compounds are compared it can be seen that carinamide produces maximum potentiation of penicillin action for approximately 2 hours whereas 'Benemid' maintains its full effectiveness for 8-10 hours. Comparisons drawn from the figures in Table I using other levels of effectiveness confirm the conclusion that the ability of 'Benemid' to support the chemotherapeutic action of penicillin is more prolonged than that of carinamide. These data indicate that when equivalent oral doses are given, 'Benemid' has at least two to three times the duration of action of carinamide.

TABLE I. Percentage Decrease of Penicillin PD_{50} Caused by Adjunct Feeding at Various Times Before Penicillin Therapy.

Hr before penicillin therapy	Carinamide		'Benemid'	
	5 mg	10 mg	5 mg	10 mg
0	90	94	95	97
1	87	94	89	98
2	64	93	90	96
4	29	57	73	92
6	11	26	65	93
8	16	37	23	91
10				85

Since the greater activity of 'Benemid' in enhancing the effectiveness of penicillin could be caused either by its longer persistence in the blood stream or by the possession of a greater intrinsic activity, experiments were undertaken to determine the plasma concentrations produced by equivalent single oral doses of carinamide and of 'Benemid'. For this purpose four large groups of mice were employed. In a series of carefully timed experiments each mouse was fed a single dose of either 5 or 10 mg of 'Benemid' or carina-

---

[‡] Obtained from Carworth Farms, New City, N. Y.

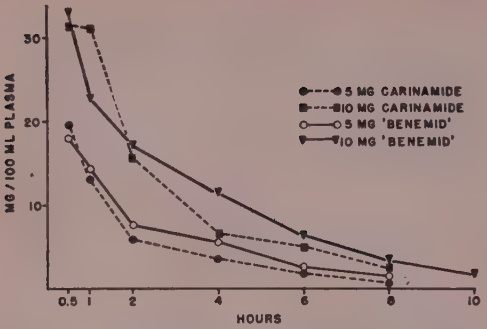


FIG. 1. Average plasma concentrations of carinamide and 'Benemid' in mice produced by single oral doses.

mide. At exactly ½, 1, 2, 4, 6, 8 and 10 hours after the feeding, 17 to 20 mice from each group were exsanguinated under ether anesthesia. Pooled plasma samples were assayed using a modification of the method of Brodie, Levy and Bernstein for the determination of carinamide(7) and both a spectrophotometric and a colorimetric method for 'Benemid'(8).§ The results of several replicated experiments were averaged and are shown in Fig. 1. It will be seen that, although the initial concentrations of equivalent doses of the two drugs are quite similar, the plasma levels of 'Benemid' fall off at a slower rate than do those of carinamide. However, this difference in the rate of disappearance from the blood stream does not appear to be sufficient to explain completely the difference in effectiveness of these compounds in the enhancement of penicillin therapy.

Table II shows the plasma concentration values of carinamide and 'Benemid' that were observed to reduce the PD_{50} of penicillin by 85%, 70%, and 40%. The times at which these percentage reductions occurred were obtained by interpolation on curves constructed from the data of Table I. The plasma concentrations listed in Table II then were read from the blood level curves at the indicated times. It can be seen that carinamide in amounts of 12.5 and 14 mg per 100 ml were required to decrease the penicillin PD_{50} 85%, whereas 'Benemid' at 7 and 1.7 mg per 100 ml accomplished the same reduction. The other figures also demonstrate that equal effects are produced by about one-half as much 'Benemid' as carinamide.

Discussion. The mouse protection data clearly indicate that the effect of 'Benemid' upon the therapeutic activity of penicillin is of considerably longer duration than is the effect of carinamide. The work of Boger, Gallagher, and Pitts on human patients(9) supports this observation.

When the protection test data are considered in conjunction with the information concerning the rate with which these two drugs disappear from the plasma, it becomes apparent that the prolonged effectiveness of 'Benemid' in mice is the result not only of its slower elimination but also of its greater intrinsic activity. Although the renal clearance studies of Beyer, *et al.*, have indicated that equal plasma concentrations of carinamide and 'Benemid' exert approximately equal effects on penicillin excretion in dogs, the data reported here indicate that, in the mouse, 'Benemid' may be at least twice as effective as is carinamide at apparently equivalent plasma concentrations. This seeming discrepancy between the results of the two types of experiment may be explainable on the basis of species differences in the manner in which these two compounds are metabolized. Clinical impressions, based on determination of the plasma concentration of these two drugs found necessary to produce enhancement of the penicillin plasma concentrations in human beings, suggest that the greater effectiveness of 'Benemid' demonstrated in mice also is found in humans(10).

Summary. 1. Oral administration of either carinamide or 'Benemid' enhances the therapeutic effectiveness of intramuscularly in-

TABLE II. Plasma Concentrations of Carinamide and 'Benemid' (mg/100 ml) Required to Produce Specified Reductions of Penicillin PD_{50}.

% reduction of penicillin PD_{50}	Carinamide		'Benemid'	
	5 mg	10 mg	5 mg	10 mg
85	12.5	14	7	1.7
70	8	10	4.5	—
40	4	5.5	2	—

---

§ These assays were performed through the kind cooperation of Dr. K. H. Beyer and Grace S. Schuchardt of the Pharmacology Section, Research Division, Sharp & Dohme, Inc.

jected penicillin in mice experimentally infected with Type I pneumococci. 2. When equal oral doses of these adjuncts are given, 'Benemid' had at least two to three times the duration of action of carinamide. 3. Plasma concentration studies show that 'Benemid' disappears from the blood stream at a somewhat slower rate than does carinamide. 4. Plasma concentration studies, combined with a consideration of the protection test data, suggest that 'Benemid' has about twice the intrinsic activity of carinamide. 5. The combined data indicate that the prolonged effectiveness of 'Benemid' in mice is the result not only of its slower rate of disappearance from the blood stream but also of its greater intrinsic activity.

---

1. Beyer, K. H., *Science,* 1947, v105, 94.

2. Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, A. K., *J. Am. Med. Assn.,* 1947, v134, 1528.

3. Verwey, W. F., and Miller, A. K., Proc. Soc. Exp. Biol. and Med., 1947, v65, 222.

4. Eisman, S. H., Kay, C. F., Norris, R. F., and Boger, W. P., *Am. J. Med. Sci.,* 1949, v217, 62.

5. Beyer, K. H., Russo, H. F., Tillson, E. K., Miller, A. K., Verwey, W. F., and Gass, S. R., *Am. J. Physiol.,* 1951, v166, 625.

6. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

7. Brodie, B. B., Levy, B., and Bernstein, E., *J. Pharm. and Exp. Ther.,* 1947, v91, 246.

8. Tillson, E. K., Russo, H. F., Schuchardt, G. S., Gass, S. R., and Beyer, K. H., 1952, to be published.

9. Boger, W. P., Gallagher, M. E., and Pitts, F. W., *J. Phila. Gen. Hosp.,* 1950, v1, 51.

10. Boger, W. P., Personal communication.

Received February 20, 1952. P.S.E.B.M., 1952, v79.

Comparative Study of Anabolic Effects of Three Androgenic Steroids.*

(19425)

W. Dean Warren and Mark A. Hayes. (Introduced by R. L. Kahn.)
From the Department of Surgery, University of Michigan Medical School, Ann Arbor, Mich.

It has been reported(1) that testosterone cyclopentylpropionate (hereafter referred to as TCP) has greater anabolic and androgenic activity in rats than testosterone propionate (TP) or methylandrostenediol (MA). Studies in man(2) have shown that TCP has a more effective androgenic action than has TP. As a result of recent clinical experiments(3), it seemed desirable to attempt a comparative evaluation of the anabolic potency in man of these 3 steroidal preparations.

Method. A 56-year-old white man in good nutrition with a gastrostomy for a benign stricture of the esophagus was placed on a constant daily tube diet supplying: protein 70 g (11.2 g nitrogen), carbohydrate 98 g, 1730 calories; sodium 55.5 meq, potassium 70.7 meq, and adequate vitamin supplements all in a volume of 3050 cc. Twenty-four-hour urine specimens were collected and were measured carefully for volume and analyzed for total nitrogen (Koch-McMeekin method), carbohydrate (Nelson-Somogyi method), sodium and potassium (Perkin-Elmer flame photometer). Periodic determinations were made of the 24-hour urinary excretion of 17-ketosteroids (Robbie and Gibson method). Fecal nitrogen was not measured and was assumed to be constant(4). After a 7-day control observation period, and at 7-day intervals, the patient was given an intramuscular injection with equivalent contents of testosterone (2) of each of the 3 steroids in the following order: TP (300 mg), MA (300 mg), and TCP (353 mg).

Results. Fig. 1 presents the metabolic data of the entire experiment graphed according to the method of Reifenstein, Albright, and Wells(5). The apparent nitrogen retention manifested during the control period is accounted for in the calculated but unmeasured fecal losses. The dotted line extending through the experimental periods is the average excretion level of each substance calculated from

---

* Supported by the Lawrence J. Montgomery Research Fund.

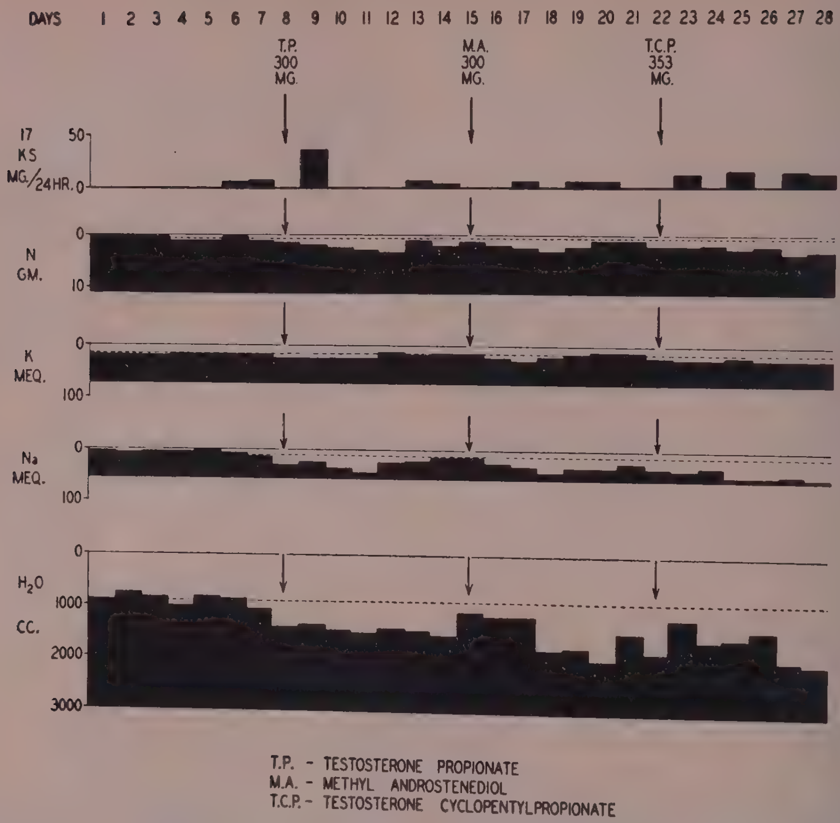


FIG. 1. Daily metabolic data illustrating the effects of the 3 androgenic drugs. The arrow indicates the day of administration of the drug. Data charted according to Reifenstein, Albright and Wells(5).

the control period and shows the absolute retentions produced by the 3 steroid preparations. Table I presents both the daily average excretory levels and the cumulative balances of water, nitrogen, sodium and potassium as effected by the 3 individual steroids compared with the control period. The anticipated retention of water, nitrogen, sodium and potassium(6,7) occurred following the injection of each of the preparations. It is readily apparent, however, (Table I) that the most profound effect followed the administration of TCP, when the comparable 7-day period cumulative retention of nitrogen was significantly greater by almost a factor of 2 and the 24-hour urinary excretion of 17-ketosteroids still indicated available steroid. The cumulative retentions of nitrogen, water and electrolytes with both TP and MA were essentially the same with equivalent dosages and were of much shorter duration, though the 17-ketosteroid excretion is reliable only in evaluating the duration of activity of TP, since MA is not metabolized and excreted as a

TABLE I. Daily Average Excretory Values and Cumulative Balances for Sodium, Potassium, Nitrogen as Influenced by the 3 Androgenic Agents.

Period, days (incl.)		Water, cc	N, g	Na, meq	K, meq
		Avg daily urinary excretion			
Control	1-7	2150	10.3	44.5	54.6
TP	8-14	1640	9.1	25.9	50.8
MA	15-21	1590	9.2	23.8	50.3
TCP	22-28	1440	8.2	11.5	48.7
		Cumulative retention			
Control	1-7	—	—	—	—
TP	8-14	3700	8.2	150.3	16.8
MA	15-21	3910	7.7	165.1	20
TCP	22-28	4990	14.1	251.4	31.1

17-ketosteroid.

Summary. Comparative anabolic evaluations after a single equivalent dose injection of testosterone propionate, methylandrostenediol and testosterone cyclopentylpropionate indicate that TP and MA are about equal in anabolic potency, while TCP is twice as effective over a comparable period of time. TCP is also effective for a greater period of time, undetermined in this study.

The authors are indebted to many people for their assistance in this investigation: B. S. Dobbie, H. Bergene, and their staff on the surgical wards, University Hospital; J. P. Chandler, Consulting Biochemist, University Hospital; H. F. Hailman, the Upjohn Co., Kalamazoo, Mich., for the oral fat emulsion (Lipomul) and the testosterone cyclopentylpropionate; F. E. Houghton, of Ciba, Summit, N. J. for the testosterone propionate; M. W. Amster, Schering Corporation, Bloomfield, N. J., for the methylandrostenediol (Methostan).

---

1. Sakamoto, W., Gordon, G. S., and Eisenberg, E., Proc. Soc. Exp. Biol. and Med., 1951, v76, 406.
2. Hailman, H. F., personal communication.
3. Hayes, M. A., Hodgson, P. E., and Coller, F. A., *Ann. Surg.*, in press.
4. Albright, F., Forbes, A. P., Bartter, F. C., Reifenstein, E. C., Jr., Bryant, D., Cox, L. D., and Dempsey, E. F., in Symposia on Nutrition, Vol. II *Plasma Proteins*, J. B. Youmans, edit., C. C. Thomas, Springfield, 1950.
5. Reifenstein, E. C., Jr., Albright, F., and Wells, S., *J. Clin. Endocrinol.*, 1945, v5, 367.
6. Kenyon, A. T., Knowlton, A., Sandiford, I., Koch, F. C., Lown, G., *Endocrinol.*, 1940, v26, 26.
7. Hayes, M. A., *Univ. Mich. Med. Bull.*, 1951, v17, 83.

Received February 21, 1952. P.S.E.B.M., 1952, v79.

Effect of Follicle-Stimulating Hormone on Gonads of Immature C57 Black Mice.* (19426)

Henry D. Moon and Choh Hao Li.

From the Division of Pathology, School of Medicine, and the Department of Biochemistry, University of California, San Francisco and Berkeley.

While mice have been utilized extensively for the bioassay of urinary gonadotropins, there have been no reliable data on the effects of highly purified follicle-stimulating hormone in mice. This study was performed with the purpose of determining the responsiveness of mice of the C57 black strain to a highly purified follicle-stimulating hormone (FSH) preparation isolated from sheep pituitary glands (1,2). Female C57 black mice, 20 to 24 days old, were injected daily with FSH for 5 days and autopsied 24 hours after the final injection. Groups of 6 mice were used for each level of FSH. At autopsy the ovaries and uteri were dissected, weighed and fixed in formalin. Paraffin sections of the uteri and the ovaries, stained with hematoxylin and eosin, were prepared.

A right orchidectomy was performed on male mice 21 to 27 days old of the C57 black strain; the right testis was weighed and fixed in formalin. Immediately following orchidectomy, FSH was injected daily for 5 days. The animals were sacrificed on the 6th day following orchidectomy, 24 hours after the final injection. The left testis was weighed and fixed in formalin. Microscopic sections of both testes were prepared. Controls of both sexes of similar age range were not injected.

Results. Female mice. The ovaries showed no morphologic changes at 0.05 and 0.10 mg levels. The uteri of all mice showed endometrial and myometrial stimulation characteristic of that produced by estrogens. The uninjected controls showed infantile ovaries and uteri (Table I). With total doses of 0.25 mg or more the ovaries showed some follicular development. A 5 mg total dose produced occasional follicular hemorrhages and early thecal luteinization.

Male mice. The uninjected controls showed

---

* Aided in part by grants from the University of California Cancer Grant, the Eli Lilly Laboratories, Merck and Co., and the Armour Laboratories.

TABLE I. Effect of FSH on Ovaries and Uteri of Immature C_{57} Black Female Mice.

No. of mice	Age at autopsy, days	Total dosage, mg	Ovaries		Uterus	
			Wt, mg	Microscopic	Wt, mg	Microscopic
14	26–31	0	6.8 (4.2-8)	Infantile	5.6 (2-8.4)	Infantile
6	29	.05	4.3 (4-4.5)	"	11 (7.8-17.8)	Slight estrogenic effect
6	29	.1	6.1 (3.4-9.3)	"	11.7 (10.6-13.4)	Same
6	27	.25	8.6 (6.2-10.3)	Medium follicles	34.4 (28.4-38.2)	Estrous
6	31	.5	7.7 (6.4-8.9)	" "	30.6 (25.4-38.2)	"
6	26	2.5	16 (11.4-19.8)	Medium and large follicles	35.3 (25.6-56.8)	"
6	27	5	28.6 (27-29.8)	Same	37.6 (34.3-41.9)	"

TABLE II. Effect of FSH on Testes of Immature C_{57} Black Male Mice.

No. of mice	Age at autopsy, days	Total dosage, mg	Right testis		Left testis		Avg wt increase, mg
			Wt, mg	Microscopic	Wt, mg	Microscopic	
9	27–29	0	26.2 (12.9-37.8)	Infantile	29.7 (13.6-38.8)	Infantile	3.5
6	33	5	36.9 (24.8-50.2)	"	44.8 (38 -56.4)	No change	7.8
6	29	12.5	26.4 (20.9-30.1)	"	38.9 (35.8-45.1)	" "	12.5

infantile testes. The left testis was slightly heavier than the right, which had been removed as a control at the beginning of the experiment (Table II). The mice receiving FSH also had larger left than right testes; moreover, the amount of gain in weight of the left over the right was greater than in the uninjected controls. No significant microscopic changes were detectable. The prostate glands and seminal vesicles showed no observable stimulation.

Comment. The ovaries of female mice of the C57 black strain showed no significant morphologic changes in response to FSH injections up to the 0.1 mg total dose level. It is of interest that estrogenic effects on the uterus occurred at low levels of FSH which did not produce morphologic changes in the ovaries. A total dose of 2.5 mg was necessary to produce significantly larger ovaries, whereas a total dose of 0.05 mg caused some estrogenic effect on the uterus. These observations indicate that increased functional activity of the ovaries precedes morphologic alterations detectable by the usual histologic methods.

Male C57 black mice were much less sensitive to injections of FSH than were the females. Although the testes of the animals receiving the hormone showed a greater gain in weight than those of the uninjected controls, no microscopic evidence of specific stimulation was present. This is in contradistinction to the results obtained with hypophysectomized male rats; in these animals FSH caused differentiation of testicular tubules, with formation of spermatozoa(3). It may be seen in Table II that a total dose of 12.5 mg induced some increase in the weight of the testes with no signs of stimulation apparent on microscopic examination. It was thought that other strains of male mice might manifest more sensitivity but results from experiments using Ce, C_3H, dba, and Webster strains at a total dose level of 1.25 mg of FSH gave no indication of testicular stimulation.

Summary. The responsiveness of mice to injections of a highly purified FSH preparation has been investigated. At a total dose level of 12.5 mg no microscopic changes in the testes of male mice were observed, although some weight increment was evident. In female mice the uterus showed greater sensitivity to hormone injections than did the ovaries.

1. Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, 1949, v109, 445.

2. Li, C. H., *Vitamins and Hormones*, 1949, v7, 223.

3. Simpson, M. E., Li, C. H., and Evans, H. M., *Endocrinology*, 1951, v48, 370.

Received February 26, 1952. P.S.E.B.M., 1952, v79.

Encephalitis in Midwest IX. Neutralizing Antibodies in Wild Birds of Midwestern States. (19427)

CLARENCE A. SOOTER, BEATRICE F. HOWITT, AND RACHAEL GORRIE.

From the Communicable Disease Center, Public Health Service, Atlanta, Ga.

In the virus diseases termed the arthropod-borne encephalitides, considerable work points definitely toward birds as biological reservoirs of these viruses, including St. Louis encephalitis, Japanese B encephalitis, and Eastern and Western equine encephalomyelitis. The literature describes the possible part birds may play as reservoirs and their possible role in the infection of man with one of these viruses(1-7). The present paper deals with wild birds and results of blood serum surveys in Colorado, Kansas, and Missouri in 1949, in which several species of birds have been sampled and tested for past infections of the St. Louis (St. L.) and Western equine encephalomyelitis (WEE) viruses. These collections were a part of an extensive search for natural reservoirs of these organisms.

Methods. Surveys were made in Kansas and Missouri in 1949 from late spring through the fall. Samples were collected in Colorado during the third week of November 1949. Species sampled and results of tests are given in Table I and Table II. The majority of the waterfowl blood samples was obtained through cooperation of the U. S. Fish and Wildlife Service personnel during banding operations at Swan Lake and Squaw Creek National Wildlife Refuges in Missouri. These blood samples were taken from the wing veins. Other bloods were taken from birds collected during special surveys in southeastern and western Kansas and in Weld County, Colorado. During the Colorado survey, turkey and chicken sera were also collected for testing. The turkeys sampled were mature-sized birds reared from poults that year and were bled at a Greeley, Colo., processing plant just prior to slaughter. The chickens were full grown and of various ages. Blood was sampled just prior to slaughter at a Greeley processing plant. Both the chickens and turkeys were from flocks in Weld County and surrounding counties. The sera were tested for neutralizing antibodies against St. L. and WEE viruses according to a modification of the methods given in the U. S. Army Laboratory Manual(8). All sera were inactivated at 56°C for 30 minutes. The serum virus mixtures were prepared in the late afternoon and held overnight in the refrigerator before they were used to inoculate mice intracerebrally. Neutralization indices of 50 or more were regarded as positive and those between 32 and 50 as equivocal.

Results. It will be noted in Table I that incidence of past infections was relatively low in the specimens collected in Kansas and Missouri, with 13 of 112 or 11.6 per cent showing evidence of having had Western equine encephalomyelitis, and no incidence of St. Louis virus in 68 specimens. It was interesting and deemed noteworthy that all of the positives in Table I were in migratory birds. Also few human or equine encephalomyelitis cases were reported during 1946 through 1949 in these states or in other localities where these birds might have been

TABLE I. Results of Neutralization Tests on Bird Sera Collected in Kansas and Missouri, 1949.

		Tested WEE	Pos. WEE	Tested St.L.	Pos. St.L.
Pied-billed grebe	*Podilymbus p. podiceps*	1	1	—	—
Canada goose	*Branta canadensis*	27	0*	25	0
Mallard duck	*Anas p. platyrhynchos*	22	3†	14	0
Swainson's hawk	*Buteo swainsoni*	4	2	1	0
Sparrow hawk	*Falco sparverius*	1	0	—	—
Eastern bob-white	*Colinus v. virginianus*	3	0	—	—
American coot	*Fulica a. americana*	5	2	—	—
Golden plover	*Pluvialis d. dominica*	2	0	2	0
Mourning dove	*Zenaidura macroura*	10	0	4	0
Red-headed woodpecker	*Melanerpes erythrocephalus*	1	0	—	—
Eastern kingbird	*Tyrannus tyrannus*	2	2	—	—
Cliff swallow	*Petrochelidon a. albifrons*	1	0	—	—
Eastern crow	*Corvus brachyrhynchos*	13	0	11	0
Eastern robin	*Turdus m. migratorius*	1	0	1	0
Meadowlark	*Sturnella neglecta*	3	0	1	0
Red-winged blackbird	*Agelaius phoeniceus*	3	0	3	0
Rusty blackbird	*Euphagus carolinus*	2	1	2	0
Brewer's blackbird	*Euphagus cyanocephalus*	3	2	1	0
Bronzed grackle	*Quiscalus versicolor quiscula*	1	0	1	0
Eastern cowbird	*Molothrus a. ater*	2	0	—	—
English sparrow	*Passer d. domesticus*	5	0	2	0
	Total	112	13 (11.6%)	68	0

* 1 was equivocal. † 2 others equivocal.

TABLE II.

A. Results of neutralization tests on bird sera collected in Colorado, 1949

		Tested WEE	Pos. WEE	Tested St.L.	Pos. St.L.
Green-winged teal	*Anas carolinense*	1	0	—	—
Pheasant	*Phasianus colchicus torquatus*	1	1	—	—
Pigeon	*Columba l. livia*	3	2	2	0
Desert horned lark	*Otocoris alpestris leucolaema*	1	0	—	—
Magpie	*Pica p. hudsonia*	11	4*	1	0
Meadowlark	*Sturnella neglecta*	3	0	2	0
Red-winged blackbird	*Agelaius phoeniceus*	3	0	1	0
English sparrow	*Passer d. domesticus*	4	0†	—	—
House finch	*Carpodacus mexicanus frontalis*	1	0	—	—
	Total	28	7(25%)	6	0

B. Results of neutralization tests on domestic fowl sera collected in Colorado, 1949

	Tested WEE	Pos. WEE	% positive	Tested St.L.	Pos. St.L.	% positive
Turkey	38	11	29	41	0	0
Chicken	23	7	30.4	21	0	0

* 2 others equivocal. † 1 was equivocal.

at some time during their migrations, wintering, or nesting activities. In contrast, all of the positive serum samples collected in Colorado (Table II) were from resident species of the area being investigated. This was significant in that a small epidemic of WEE had occurred just previously in people and horses in this part of Colorado. This pointed toward local sources of virus infection in nature and was supported by field work in this investigational area the following summer (1950). During that season, nestling bird bloods containing WEE virus were collected on 3 different occasions(12).

The presence of WEE neutralizing antibodies in 29% of the turkeys and 30.4% of the chickens (Table II B) also indicated local sources of virus.

Discussion. The above results support past references that birds are involved in WEE outbreaks. The exact role they play is not as yet known. The host range of these encephalitis viruses among bird species in nature is known only partially and future surveys and investigations will undoubtedly continue to add to present lists. In the Missouri-Kansas investigational area we know that 7 species (Table I) may be involved. These represent only a small percentage of the several hundred species of birds that frequent this region at some time during the year. In Colorado four of the approximately 365 species and subspecies frequenting this area have been found with positive neutralizing antibodies for WEE or with a viremia for WEE (Table II)(12, 16).

Surveys of this type are deemed important in that they reveal evidences of past inapparent infections and presence of virus in nature when few or no human or horse cases occur. Often bird infections go undetected because evidence of St. L. and WEE infection in domestic and wild birds is usually characterized by a lack of any apparent clinical evidence of infection(13). Conversely, Eastern equine encephalomyelitis is known at times to be fatal to a high percentage of some of our common birds(7-11). Results from these serum surveys are also of value for correlation with findings of blood sucking parasites of birds being infected with WEE and St. L. viremia(7,14,15).

Comment. This information is given to emphasize the possible importance of birds in outbreaks of the above-mentioned arthropod-borne encephalitides, as well as to help prevent possible misinterpretation of findings relative to various bird species.

Summary. Serum surveys for St. Louis and WEE neutralizing antibodies in migratory and resident wild birds in midwestern states during 1949 showed 11.6% of birds with positive WEE neutralizing antibodies in Kansas and Missouri area where little or no encaphalitis occurred. This is compared with a survey in Colorado during 1949 following a small WEE epidemic when the sera of 25% of resident wild birds showed positive WEE neutralizing antibodies.

---

1. Howitt, B. F., and Van Herick, W., *J. Inf. Dis.*, 1942, v71, 179.

2. Reeves, W. C., Proc. 49th Ann. Meet. U. S. Livestock Assn., Dec. 1945, p150.

3. Cox, W. R., Jellison, W. L., and Hughes, L. E., *Pub. Health Rep.*, 1941, v56, 1905.

4. Hammon, W. McD., Reeves, W. C., and Sather, Gladys E., *Am. J. Hyg.*, 1951, v53, 249.

5. Hammon, W. McD., *Am. J. Trop. Med.*, 1947, v28, 515.

6. ———, Proc. and Papers 16th Ann. Conf. Calif. Mosquito Abatement Assn., Feb. 1948, 4.

7. Davis, W. A., *Am. J. Hyg.*, 1940, v32, 45.

8. Simmons, J. S.. and Gentzkow, C. J., *Laboratory Methods of the U. S. Army,* Lea and Febiger, Phila., 1944.

9. Van Rockel, H., and Clarke Miram K., *J. Am. Vet. Med. Assn.*, 1939, v94, 466.

10. Beaudette, F. R., and Hudson, C. B., *J. Am. Vet. Med. Assn.*, 1945, v107, 384.

11. Beaudette, F. R., and Black, J. J., *J. Am. Vet. Med. Assn.*, 1948, v112, 140.

12. Sooter, Clarence A., Howitt, B. F., Gorrie, Rachael, and Cockburn, T. Aidan, PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 393.

13. Reeves, William C., *Am. J. Pub. Health,* 1951, v41, 678.

14. Hammon, W. McD., Reeves, W. C., Cunha, R., Espana, C., and Sather, G., *Science,* 1948, v107, 92.

15. Miles, Virgil I., Howitt, Beatrice F., Gorrie, Rachael, and Cockburn, T. Aidan, PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 395.

16. Niedrach, Robert J., and Rockwell, Robert B., *The Birds of Denver and Mountain Parks.* The Colo. Museum of Nat. Hist. Popular Series No. 5. Denver. 1939.

Received February 25, 1952. P.S.E.B.M., 1952, v79.

Effect of Antibiotics on Development of Resistance to Hemolytic Streptococcus Infection.* (19428)

R. S. SPECK AND E. JAWETZ.

From the Division of Microbiology, University of California School of Medicine, San Francisco.

It is current medical practice to administer early, intensive chemotherapy in all respiratory infections ascribed to hemolytic streptococci in part at least to prevent the possible development of the hypersensitive state. The question arises whether such treatment might also affect the development of resistance to bacterial infection and thus alter the natural history of streptococcal disease. A laboratory model has been employed to investigate this point.

A recent report from this laboratory(1) described a subacute infection in mice induced by the inoculation of beta hemolytic streptococci into the thigh muscle(2). This infection was characterized by the development of a progressive, hard swelling at the site of infection for 4 or 5 days, then dissemination of bacteria throughout the body, toxemia, and death between the 5th and 8th day after inoculation. The mortality in untreated groups was about 95% with death occurring in all animals which had developed a local lesion. Treatment with antibiotics reduced the death rate in proportion to the effective dose of the drug. Surviving mice usually developed some induration of the thigh early in the treatment period. In some animals this local lesion regressed during therapy, in others it persisted and several weeks later streptococci could be cultured from the muscle lesion. When such survivors were challenged with an otherwise lethal intraperitoneal infection with homologous organisms they survived in significant numbers. The present report describes an experiment which was undertaken to evaluate this phenomenon.

Experimental. White Swiss mice from a heterologous stock weighing from 20 to 25 g were used. The infection was produced by the beta hemolytic, group A, type 3 streptococcus strain C203 used in previous work (1,2). The bacteria were grown in 5% sheep blood broth; virulence was maintained by weekly passage through mice. The LD50 for intraperitoneal infection was 0.2 ml of a 10^{-6} dilution of an 18-hour culture containing about 60-100 organisms or chains. Commercial crystalline potassium penicillin G was dissolved in sterile 0.85% sodium chloride solution to make a stock solution of 100,000 units/ml which was stored at 4°C during the treatment period. Aureomycin hydrochloride (Lot 7-6116), supplied by Dr. S. S. Hardy, Lederle Laboratories, was dissolved in 0.85% sodium chloride solution and sterilized by Seitz filtration to make a stock solution of 2.5 mg/ml which was quick-frozen and stored at —20°C. Suitable saline dilutions for injection were prepared daily from the stock solutions. Mice were infected by injecting 0.1 ml of a 10^{-4} dilution in broth of an 18-hour culture of the streptococcus into the left thigh. Treatment with antibiotics was started 24 hours after infection and continued at 24-hour intervals for 5 doses. The dose of antibiotic was administered intraperitoneally in a volume of 0.2 ml. Four groups of 100 mice each received respectively a total of 150 and 1500 μg of penicillin and 250 and 2500 μg of aureomycin. A control group of 20 mice was left untreated. All animals were observed for 16 days after infection or 11 days after the last treatment, and deaths were recorded. In the survivors the persistence at the site of infection of an indurated lesion was noted and those demonstrating it were marked with a dye. All the survivors were then challenged with an intraperitoneal inoculation of 0.2 ml of a 10^{-4} dilution in broth of an 18-hour culture of the homologous streptococcus strain. This dose contained about 100 LD50's and was known from previous experience to give a mortality of 95-100%. Following this chal-

---

* Supported in part by a grant from the Committee on Research of the University of California School of Medicine.

TABLE I. Persistence of Streptococci Related to Resistance to Subsequent Homologous Challenge Infection.*

	Initial subacute intramusc. infection				Survivors challenged with acute intraper. infection			
			Survivors with lesions			Survivors		Survivors with lesions
Treatment over 5 days	Infected No.	Survivors No.	No.	%	Infected No.	No.	%	No.
Penicillin, total 150 µg	100	67	32	47.8	67	30	44.8	21
Aureomycin, total 250 µg	100	80	13	16.2	80	16	20	11
Penicillin, total 1500 µg	100	78	7	9	78	14	17.9	6
Aureomycin, total 2500 µg	100	96	6	6.3	96	4	4.2	3
Control for initial infection	20	0	—	—	—	—	—	—
Control for challenge infection	—	—		—	25	0	.0	—

* Mice were infected intramuscularly with beta hemolytic streptococcus strain C203 and treated with the specified antibiotic 5 days; 16 days after the first infection survivors were challenged with an intraperitoneal inoculation of the homologous strain.

lenge infection the mice were observed for 7 days and deaths were recorded. Survivors were rechallenged twice in the same manner at intervals of 7 days. Groups of 20 normal mice were included as controls with each challenge infection. Statistical significance of the results was calculated by the χ^2 method.

Results. The relationship of antibiotic treatment resulting in survival of the initial subacute infection to the subsequent resistance to homologous reinfection is summarized in Table I. The drug schedules are arranged in order of the increasing curative effect on the initial infection: penicillin 150 µg $<$ aureomycin 250 µg $=$ penicillin 1500 µg $<$ aureomycin 2500 µg. The differences in survival rates are significant ($p < 0.01$) between the least effective dose, the intermediate doses, and the most effective dose. Of the 67% of animals surviving with 150 µg of penicillin about half showed persisting lesions, whereas of the 96% alive after treatment with 2500 µg of aureomycin only 6% had such evidence of persisting local infection. All untreated animals died.

The challenge infection was administered intraperitoneally 16 days after the initial infection. All normal control mice died. In the animals infected with the streptococcus for the second time survival of the challenge appeared to be inversely related to the efficacy of a given drug schedule in eradicating the organisms from the animal. Thus in the aureomycin treated group in which 96% had survived and only 6% developed local lesions with the initial infection, all but 4% proved to be completely susceptible to the homologous reinfection. It is significant that 3 of the 4 survivors were among the 6 animals which had local lesions ($p = 0.01$). Conversely resistance to the challenge was considerable (44.8%) in the group where treatment with a small dose of penicillin had saved only 67% of the animals permitting the development of gross local lesions in half of them. In the intermediate groups also the survival from the challenge appeared to be roughly proportional to the development of local lesions during the initial infection.

The survival rates of animals subjected repeatedly to homologous challenge are shown in Table II. The first reinfection apparently weeded out the susceptible animals so that the resistance offered to the subsequent reinfections by the surviving groups appears more solid.

One group of 20 normal mice was given the challenge intraperitoneal infection followed in 3 hours by treatment with 120 µg of penicillin intraperitoneally in 0.2 ml of saline solution.

TABLE II. Increasing Resistance to Homologous Challenge as a Result of Repeated Infection.

	First challenge*			Second challenge			Third challenge		
Mice	Infected No.	Survivors No.	%	Infected No.	Survivors No.	%	Infected No.	Survivors No.	%
Survivors of previous infection	321	64	19.9	64	44	68.8	44	37	84.1
Control	25	0	.0	—	—	—	—	—	—
,,	—	—	—	20	0	.0	—	—	—
,,	—	—	—	—	—	—	20	0	.0

* See Table I.

Seven days later the challenge infection was repeated on the 15 mice which survived the first infection. All of these mice died of the reinfection within the same time interval in which the groups of 20 normal control mice died when infected in the same manner. This confirms previous experience that mice given an acute intraperitoneal infection and treated with antibiotics within a few hours of this infection did not acquire resistance to reinfection.

Discussion. The experiment demonstrates that the less effective the treatment of the initial subacute streptococcal infection is, provided it permits significant numbers of survivors, the greater is the resistance of such survivors to subsequent homologous challenge. Correlated with this increased ability to withstand challenge is the presence of a chronic lesion at the site of injection from which streptococci can be regularly cultured. The significance of this persisting lesion is further demonstrated by the fact that among 321 animals challenged 58 or 18% had local lesions, whereas in the 64 survivors of the challenge such lesions occurred in 41 or 64% ($p < 0.01$). Thus it appears that antibiotic treatment which rapidly and completely eliminated the infecting microorganism interfered significantly with the development of homologous immunity.

It is of interest in this connection to note that the rapid and complete elimination of hemolytic streptococci from the respiratory tract of man significantly interferes with the production of antibodies(3-5). If the intensive administration of chemotherapeutic agents in streptococcal infections of man should likewise effectively prevent the development of antibacterial immunity as well as the hypersensitivity, the natural history and epidemiology of streptococcal disease might be significantly altered in the future(6).

Summary. A subacute infection of mice with hemolytic streptococci was treated with antibiotics in such a way as to interfere to a varying degree with the persistence of the bacteria in the tissue. Striking differences were noted in the subsequent susceptibility of survivors to a homologous challenge. The acquired resistance of the animals was inversely proportional to the efficacy of the treatment regimen in rapidly eliminating the infecting microorganisms in the initial infection.

---

1. Speck, R. S., and Jawetz, E., *Am. J. Med. Sci*, 1952, in press.

2. Eagle, H., Fleischman, R., and Musselman, A. D., *Ann. Int. Med.*, 1950, v33, 544.

3. Rantz, L. A., Boisvert, P. L., and Spink, W. W., *Science*, 1946, v103, 352.

4. Kilbourne, E. D., and Loge, J. P., *J. Clin. Invest.*, 1948, v27, 418.

5. Daikos, G., and Weinstein, L., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 160.

6. Rantz, L. A., *The prevention of rheumatic fever*, 1952, Charles C. Thomas.

Received February 25, 1952. P.S.E.B.M., 1952, v79.

Renal Excretion of Lanatoside C. in the Rat.* (19429)

René Bine, Jr., Shirley St. George, and Meyer Friedman.
From Mount Zion Hospital, Harold Brunn Institute for Cardiovascular Research, San Francisco, Calif.

Previous studies(1,2) have been performed to determine, by means of the embryonic duck heart method, the urinary excretion of digitoxin in the rat. It was felt desirable to determine in a similar fashion, the excretion of Lanatoside C. in the urine of the rat in order that the rate of excretion of both drugs might be compared in this species.

Methods. Various known amounts of Lanatoside C. (10, 20, 30, 40, and 60 μg) were added to 24-hour urine collections of rats. The entire sample was evaporated to approximately 2 ml, adsorbed on 5 g of diatomaceous earth and dried at 70°C *in vacuo* for 12 hours. The glycoside was eluted with 50 ml re-distilled dioxan for 2 minutes in a Waring Blender, filtered and evaporated to dryness, then taken up in 50 ml of Tyrode's solution by shaking for 1 hour. The embryonic duck hearts were then exposed to this latter solution and the time of "digitalis effect"(1) observed. A quantitative differentiation could be made between these various urine samples containing different amounts of added Lanatoside C. As little as 10 μg in a 24-hour urine sample could be detected. After these standards had been prepared, 28 male rats (Long-Evans strain) whose average weight was 209 g, were given 1 μg Lanatoside C. per g of body weight by intravenous injection. Their urine then was collected for 24 hours by placing them in plastic cages(3) specially designed for collection of urine. The urine samples of 11 of these rats were also collected on the second day following injection. All urine samples were extracted and tested on the duck hearts as described above.

Results. Twenty-eight rats after the average injection of 209 μg of Lanatoside C. excreted an average of 35 μg (Range: 10-60 μg) of Lanatoside C. or 17% of the injected amount, in the first 24-hour urine. No Lanatoside C. (*i.e.* less than 5 μg) however, was detected in urine samples collected during the second 24-hour period after injection of the drug.

Discussion. The present results indicate that the rat excretes almost 6 times as much Lanatoside C. as digitoxin(2) in his urine during the first 24 hours after injection of the glycoside. The marked difference in the excretion of the two drugs might have been expected in view of the previously discovered differences in the behavior of the two drugs in the blood of this animal. Thus, although digitoxin is strongly bound to plasma albumin, Lanatoside C. is not(4,5), perhaps explaining why the latter disappears so rapidly from the blood(6) and appears in greater quantity in the urine.

Despite this *comparatively* large renal excretion of Lanatoside C., the actual fraction disposed of in this way is only about 17% of the administered dose. The greater part of the dose then is excreted or destroyed in some other manner than by simple renal excretion of unchanged Lanatoside C.

Conclusions. A method for the quantitative determination of minute amounts of Lanato-

TABLE I. The Renal Excretion of Lanatoside C.

		Urine (0-24 hr)			Urine (24-48 hr)			
No. of rats	Avg amt Lanatoside C. inj., μg (1 μg/g body wt)	Vol, ml	Lanatoside C., μg	% excreted	No. of rats	Vol, ml	Lanatoside C., μg	% excreted
28	209	12	35	17	11	12	N.D.*	—
Range:	(137–265)	(5–17)	(10–60)	(5–29)	—	—	—	—
S.E. mean:			± 2.7	± 1.2	—	—	—	—

* None detected.

---

* This work was aided by grants from The Life Insurance Medical Research Fund, and The U. S. Public Health Service.

side C. in rat urine is described. The renal excretion of Lanatoside C. in the rat is considerably greater than that of digitoxin.

The authors wish to express their thanks to Tadashi Ishida for his valuable technical assistance.

---

1. Friedman, M., and Bine, R., Jr., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 162.

2. Friedman, M., Bine, R., Jr., and Byers, S. O., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 406.

3. Bollman, J. L., *J. Lab. and Clin. Med.*, 1948, v33, 1348.

4. Rothlin, E., and Kallenberger, A., *Arch. internat. pharmacod.*, 1950, v81, 520.

5. Bine, R., Jr., and Friedman, M., *Am. J. Med. Sciences*, 1948, v216, 534.

6. ———, *Circulation*, 1950, v1, 1182.

Received February 25, 1952. P.S.E.B.M., 1952, v79.

Complement-Fixation Studies with Poliomyelitis in Humans.* (19430)

MORRIS POLLARD.

From the Department of Preventive Medicine and Public Health, University of Texas Medical School, Galveston.

Specific complement-fixing (CF) antigens have been processed from cotton rat tissues infected with Lansing poliomyelitis virus (1-3). The CF antigens prepared by Casals and Olitsky(4,5) from MEF_1 infected tissues of new-born mice have appeared to cross somewhat with serum from Brunhilde-immune monkeys. More recently(6), specific CF antigens have been processed from nerve tissues of rhesus monkeys infected with Brunhilde and with Leon prototype poliomyelitis viruses. The latter antigens have been tested thus far only with serums from monkeys immunized with virus and the adjuvant recommended by Salk(7). The chronological antibody response of Lansing-immunized cotton rats appeared to follow a pattern in which the CF test was transitory, whereas the neutralizing property of their serum was of longer duration(1,2). The exact duration of the latter effect is uncertain but is thought to last for years. Some evidence has indicated that immunized monkeys may demonstrate a similar chronological antibody pattern.

When human cases of poliomyelitis recover from the infection, specific neutralizing antibodies rapidly appear in the blood(8,9). There has been suggestive evidence that such infection may elicit a non-specific rise of Lansing neutralizing antibody, even though the infection was of non-Lansing type(9). Lansing CF antibody has been demonstrated in human serums, but their specificity and clinical interpretation have not been studied extensively(1,5). The possibility of a non-specific rise in Lansing CF antibody during the course of infection with non-Lansing virus has not been studied. It is the purpose of this paper to describe the Lansing CF antibody response elicited in the human following infection with poliomyelitis virus.

Methods. Antigen. CF antigens were processed from Lansing poliomyelitis-infected nerve tissues of mature cotton rats (LCR) by two technics: One by precipitation of clarified nerve tissue suspensions with methanol and use of the final eluate as antigen(2); and secondly, by sedimentation of the virus in the ultracentrifuge and use of the resuspended sediment as antigen(3). The antigens were tested for specificity with antiserums prepared against Brunhilde and against Leon poliomyelitis, as well as against rabies and St. Louis encephalitis viruses. The LCR CF antigens processed by the ultracentrifugation technic appeared to be superior to those prepared by methanol precipitation because of the constancy of results and because such antigens were sufficiently sensitive to detect CF antibodies in immunized monkeys. *Serum.* Serum specimens were collected aseptically from 131

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

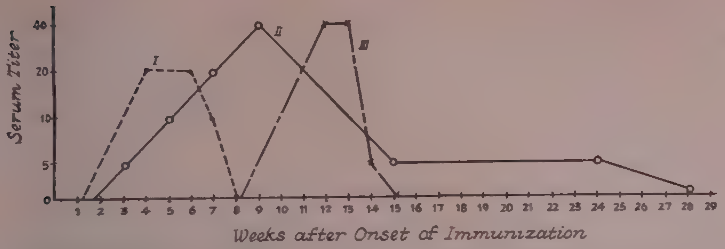


FIG. 1. Complement-fixing antibody response of Lansing + adjuvant immunized rhesus monkeys to Lansing antigen.

paralytic poliomyelitis cases in humans:[†] Eighty-five were collected 2 weeks after onset of illness; and 46, at onset and at weekly intervals thereafter, when possible. Serum specimens were collected at weekly intervals for 6 months from rhesus monkeys which were immunized by 3 weekly I.M. injections of Lansing virus plus adjuvant[‡] emulsion. All serums were stored in screw-top vials at –20°C. When prepared for CF test they were diluted 1 in 5 with 0.15 M NaCl solution and heated at 61°C for 20 minutes. *Complement-fixation test.* Antigen, antiserum (1:5) and complement (2 units) were mixed in 0.2 cc quantities each and incubated in the refrigerator overnight. Hemolysin (2 units) and 2% washed sheep red blood cells (0.2 cc each) were added to each tube and incubation continued for 30 minutes at 37°C, after which the reactions were recorded. The reactions were generally clear cut and 1+ or 2+ reactions were seldom encountered. Reactions read as 3+ and 4+ were recorded as positive. Serum dilutions were mixed with constant dilutions of antigen and 2 units of complement in order to determine their end-point titers. Each CF test was repeated at least twice. *Neutralization test.* Undiluted serum was added to an equal amount of Lansing virus which was so diluted that each inoculum contained approximately 100 LD_{50} of virus. After incubation of the serum-virus mixtures overnight in the refrigerator, eight mature Swiss mice were inoculated intracerebrally with 0.03 cc of the mixture. After 21 days of observation, if 6 or more mice survived, the test was positive; if 3 to 6 survived, the test was equivocal; if less than 3 survived, the test was negative.

Results. When monkeys were inoculated with Lansing virus in adjuvant their CF antibody response was transitory but followed a similar curve pattern (Fig. 1). One showed a rapid rise and decline, one a delayed rise and quick decline, and a third one a rapid rise and slow decline. The latter monkey was positive for 6 months and was negative thereafter. Each of 3 monkeys similarly immunized against Brunhilde and 3 against Leon viruses were negative for Lansing CF antibodies during the 6 months examination period.

Eighty-five of the specimens of human serum were collected at approximately two weeks after onset of poliomyelitis symptoms. They were tested both for MEF_1 neutralizing and for CF antibodies, the latter with methanol precipitated MEF_1 antigen. Fig. 2 indicates that 38 of these were positive and 47 negative for neutralizing effect on MEF_1 poliomyelitis virus. None of these negative serums contained CF antibodies for the same virus, while 11 of the former were positive in the 1:5 dilution of serum examined.

Forty-six specimens of serum were collected from paralytic poliomyelitis cases immediately after admission to the hospital and at weekly intervals thereafter, when possible. They were examined for CF antibodies with LCR CF antigen processed by ultracentrifuga-

---

[†] We are indebted to Drs. Herbert Spencer and Eva Maria Pfeiffer, Southwestern Poliomyelitis Respiratory Center, Houston, Texas, for generous assistance in collection of serum specimens, and for having supplied us with the clinical diagnosis of each case. Serum specimens were also kindly supplied by Dr. A. G. Bower, Los Angeles General Hospital, Los Angeles, Calif.

[‡] "Pendil" manufactured by Endo Products, Richmond Hill, N. Y.

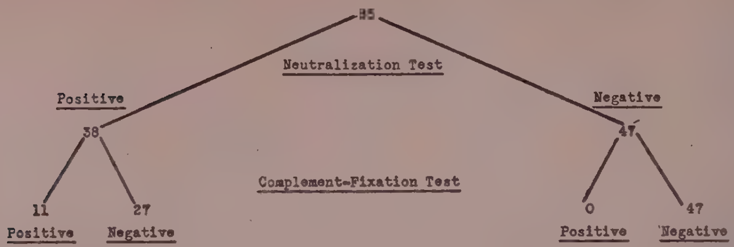


FIG. 2. Serologic studies on 85 poliomyelitis cases with MEF_1 virus. Serum collected two weeks after onset of illness.

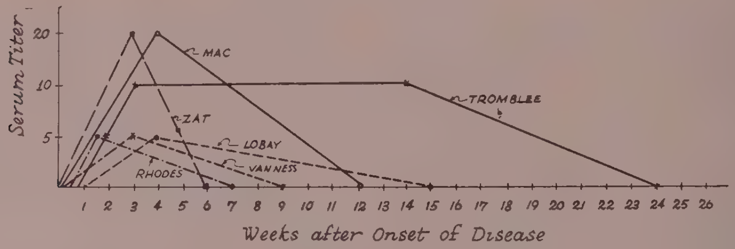


FIG. 3. Complement-fixing antibody response of paralytic poliomyelitis cases to Lansing antigen.

tion. Ten were positive and 6 of these are outlined in Fig. 3. The CF antibody titer appeared to reach its peak within the first 4 weeks of illness and declined thereafter. One case was positive during the first week, 3 during the second and third week, and one during the fourth week. The latter case could not be tested earlier. The CF reactions remained positive from 6 weeks to 3½ months after onset. The latter case was not tested between the 3½-month period and 6 months, but became negative for Lansing CF antibody some time between these two periods. The remaining 36 cases in this group of paralytic poliomyelitis were negative for LCR CF antibodies during the acute and convalescent stages of infection.

Discussion. It is apparent from the results described above that the LCR CF responses of Lansing-immunized monkeys and of naturally infected humans are of transitory nature. One might attribute the protracted curve of Monkey II (Fig. 1) to the effect of adjuvant mixture with the virus inoculum, but the CF curves of human case "Tromblee" (Fig. 3) appears to be protracted without such adjuvant being involved. Then, too, monkeys I and III exhibited shorter curves and their inocula and bleeding schedules were the same as for monkey II. We may thus be justified in saying that with the antigens used, the CF responses ranged from brief rises of 3-4 weeks duration to prolonged rises of 3-4 months. This strengthens the opinion previously suggested(*2,3*) that a positive LCR CF test might indicate current or recent Lansing infection, and that a negative CF test indicates either a remote exposure to the virus or none. The latter problem may then be unraveled by a virus neutralization test with the serum, a procedure which has become more feasible through application of the tissue culture technics of Enders *et al.*(10). It should be emphasized that the CF titer curves of Fig. 3 represent test points, joined at intervals of several weeks because the intervening specimens were unavailable. While the positive CF reactions would not be longer than demonstrated certainly they could be shorter, particularly in those cases designated as "Mac" and "Tromblee." If Fig. 3 accurately depicts the rise of LCR CF antibody in the human, then a specimen of serum collected 4 weeks after onset of illness should be the optimal period for the CF test. If a non-Lansing infection engenders a non-specific Lansing antibody response, this was not apparent in the 36 cases which were LCR CF negative during the entire period of examination. We must not insist that the LCR CF reactions here demonstrated in human serums mean that Lansing virus caused the paralytic syndrome. Since the CF antigens were prototype specific, the antibody which it demonstrated was undoubtedly Lansing induced. Therefore, these human cases were either Lansing infections or dual infections with a non-Lansing virus.

In the 85 human cases tested (Fig. 1) a CF antibody pattern was not determined, but one may note a correlation between CF and neutralizing antibody. The former was positive only among those serums having the latter antibody. Serums, negative for MEF_1 virus neutralizing effects, did not contain CF antibodies. The neutralizing antibody response of infected humans was rapid(9). In immunized cotton rats, both neutralizing and CF antibodies appeared almost simultaneously (2). If we indulge in an extrapolation of the latter data, a positive CF and negative neutralization test might be an unexpected finding in human serum.

It is not felt that the CF technic described above would be of great value in determining the past history of infection in a population survey; however, one might speculate as to its use in evaluating either prophylactic or therapeutic trials of vaccines or drugs, in identifying Lansing strains of virus, and possibly in establishing a clinical diagnosis of human Lansing infection. Further studies should delineate more accurately the CF pattern of response to infection. The CF antigens used above were prototype specific, and for such a test to be of added value to the investigator the Brunhilde and Leon prototype CF antigens must be developed further. Thus far, they are difficult to prepare routinely, probably due to the low titer of virus in the infected tissue from which such CF antigens have been processed.

Summary. The Lansing complement-fixing (CF) antibody response to immunization in monkeys and to infection in humans appears to be transitory. One hundred thirty-one paralytic poliomyelitis cases in humans were examined for CF antibody to Lansing virus. In the serums of 85 cases, collected 2 weeks after onset of clinical disease, 11 had both neutralizing and CF antibodies. CF antibody was not found in any of 47 cases which were negative for neutralizing antibody. In a series of serum specimens collected from 46 other cases, CF antibody appeared in 10. The CF antibody rose quickly and remained elevated for from 6 weeks to 3 months. A positive CF test may indicate recent infection; a negative, either no exposure or infection at a period remote in the past.

---

1. LaHelle, O., Proc. Soc. Exp. Biol. and Med., 1951, v54, 391.

2. Pollard, M., *J. Immunology,* 1951, v67, 463.

3. ———, Proc. Soc. Exp. Biol. and Med., 1951, v78, 388.

4. Casals, J., and Olitsky, P. K., Proc. Soc. Exp. Biol. and Med., 1950, v75, 315.

5. Casals, J., Olitsky, P. K., and Anslow, R. O., *J. Exp. Med.,* 1951, v94, 123.

6. Pollard, M., Hsiang, C. M., and Sharp, G. R., Proc. Soc. Exp. Biol. and Med., 1952, v79, 48.

7. Salk, J. E., Lewis, L. J., Bennett, B. L., and Youngner, J. S., *Am. J. Hygiene,* 1951, v54, 157.

8. Hammon, W. McD., and Roberts, E. C., Proc. Soc. Exp. Biol. and Med., 1948, v69, 256.

9. Steigman, A. J., and Sabin, A. B., *J. Exp. Med.,* 1949, v90, 349.

10. Enders, J. F., Weller, T. H., and Robbins, F. C., *Science,* 1949, v109, 85.

Received March 4, 1952. P.S.E.B.M., 1952, v79.

Effects of Fluoride on Developing Bones in Tissue Culture. (19431)

GEORGE H. PAFF AND M. JOHN BOYD.

From the Departments of Anatomy and Biochemistry, Hahnemann Medical College, Philadelphia, Pa.

Among the significant contributions to the study of calcification in osteogenesis are two observations by Gutman and his colleagues (1,2): first, calcifying cartilage contains a phosphorylative glycogenolytic enzyme system which is capable of synthesizing potential substrates for bone phosphatase; second, if the synthesis of substrates is interfered with by sodium fluoride, calcification can be completely inhibited.

The first observation was based on a study of digests of minced epiphyseal cartilage; the second on a study of slices of epiphyseal cartilage incubated for a brief time in solutions of known chemical composition. Since the technics employed do not permit of tissue survival, the present work was undertaken to determine if NaF can inhibit the calcification process in growing bones without influencing the growth of tissue. An affirmative answer could be offered as additional evidence to disprove the current concept that glucose-1-phosphate is a substrate for bone phosphatase(3).

Methods. Metatarsals and fibulas from 9- to 10-day-old chick embryos were chosen for study because they can be grown *in vitro* with ease and because chemical analysis of embryos in this age range yield glycogen and the intermediate products of its phosphorylation(4). Pairs of corresponding bones were isolated and grown for 4 to 5 days upon the surface of clotted media consisting basically of equal parts of blood plasma and embryonic extract. One member of each pair of bones was grown on media containing NaF, the other member was grown on media free of NaF as a control. Before placing the isolated bones on the clotted media they were washed in embryonic extract which, in the case of those to be grown on fluoride, contained NaF at a concentration equal to twice that of the clotted media. Twelve pairs of bones were studied at each of the following concentrations of NaF: 1-20,000, 1-10,000, 1-5000, 1-1000, 1-500 and 1-200 M. Metatarsals were used for the 1-20,000 and 1-10,000 M experiments. For all others we used fibulas. Because of the possibility of undesirable osmotic effects the medium for the controls contained sufficient NaCl to compensate for the amount of NaF added to the medium for the experimental bones. The increase in length of the bones was determined from measurements of the overall lengths recorded at the start and at the end of the observation period. The amount of calcification was determined by measuring the length of the zones of mineralization visualized by staining the bones with alizarin red S at the end of the experiment(5). The amount of connective tissue growth was estimated by observing the extent of the fibroblastic halo. Finally, a record of the general appearance of each preparation was kept.

Results. Most of the pertinent data for 72 pairs of bones is summarized in Table I. Each value represents the average measurement for 12 bones. By comparing the various measurements of the bones grown on NaF with the measurements of the controls it can be seen that at concentrations of 1-20,000, 1-10,000, 1-5000 and 1-1000 M the NaF did not exert an effect upon either the increase in length of the bones or upon the degree of

TABLE I. See Text for Explanation.

Conc. of NaF	Overall length, mm		Zone of mineralization, mm
	Start	End	
	Sodium fluoride		
1-20000 M	4.72	6.08	1.60
1-10000	4.29	5.75	1.60
1- 5000	3.83	4.51	1.38
1- 1000	3.67	4.24	1.25
1- 500	3.48	4.12	1.00
1- 200	3.47	3.98	.87
	Controls		
1-20000	4.75	6.01	1.60
1-10000	4.29	5.74	1.61
1- 5000	3.85	4.49	1.40
1- 1000	3.65	4.19	1.25
1- 500	3.48	4.14	1.30
1- 200	3.48	4.16	1.21

calcification, as determined by the length of the zone of mineralization. Only one difference was observed betwen the fluoride treated bones and their controls in these weaker concentrations. In many of the bones grown on fluoride the area of calcification was distinctly darker and more sharply defined than in the controls. This difference became apparent as early as the second day of the experiment and sometimes became more pronounced as time elapsed.

At 1-500 M NaF there was still no difference in the growth of the fluoride treated bones and their controls. The treated bones had grown 0.64 mm in length (3.48 to 4.12 mm) while the controls had grown 0.66 mm in length (3.48 to 4.14 mm). In contrast to this however, a marked difference in the degree of calcification between the fluoride treated and control bones appeared. Thus, the length of the zone of mineralization in the fluoride treated bones was only 1.00 mm while in the corresponding controls it was 1.30 mm.

At the highest concentration of NaF studied, namely 1-200 M the first indication of inhibition in growth was obtained. The fluoride treated bones grew only 0.51 mm in length (3.47 to 3.98 mm) while the controls showed a growth increase of 0.68 mm (3.48 to 4.16 mm). Accompanying this, there was a marked difference between the length of the zone of mineralization in the fluorine treated (0.87 mm) and control (1.21 mm) bones.

No obvious difference in the size of the fibroblastic halo was observed between the control and the fluoride treated bones until the concentration of NaF had reached 1-200 M. At this level the fluoride had a retarding influence on the development of the connective tissue halo.

Discussion. Before analyzing the results it should be understood that the metatarsals and fibulas of 9- to 10-day-old chick embryos are "bones" in the cartilage miniature stage. The "overall" length of the "bones" given in Table I are really a practical indication of the amount of cartilage which is present at the beginning and at the end of the experiment. The calcification which occurs involves only cartilage. The so-called zone of mineralization is the area of calcified cartilage which is visualized by staining with alizarin red S. In addition, metatarsal bones at this age are actually longer than are fibulas. This explains why the various measurements are greater at 1-20,000 and 1-10,000 M NaF than they are in the higher concentrations. Metatarsals were used at the two weakest dilutions.

The results themselves demonstrate that NaF, at a concentration which does not disturb growth, can seriously interfere with the calcification process. This confirms and extends the observations of previous workers (1,2,6) and emphasizes the importance of phosphorylative glycogenolysis in relationship to calcification. Sodium fluoride is known to inhibit enolase, the enzyme immediately involved in the production of phospho-pyruvate. Since calcification was stopped by the fluoride, the conclusion seems justified that phosphorylation of glycogen probably progresses to the phosphopyruvate stage before a suitable substrate for bone phosphatase is produced.

These findings are not in agreement with the current idea that glucose-1-phosphate is a substrate for bone phosphatase(3). Phosphorylase, the enzyme involved in the production of glucose-1-phosphate is not inhibited by NaF. In this connection we tried, without success, to interfere with calcification by using phloridzin and phloretin (known inhibitors of phosphorylase). Both compounds are practically insoluble in our media and are too toxic to permit of study in concentrations which could be expected to inhibit phosphorylase while not killing the bones.

Another possible factor in the stoppage of calcification by NaF must be considered. Since the fluoride might compete with the phosphate for calcium, perhaps fluoride acts by binding calcium. Thus, even concentrations of NaF which have no apparent effect upon growth or calcification cause a reaction within the zone of mineralization which renders it visibly different from the same zone in the controls. Finally it is probably significant that whereas Robison and Rosenheim (6) and Gutman, Warrick and Gutman(2) had been able to inhibit calcification in 1-

10,000 M NaF, we found it necessary to increase the fluoride concentration to 1-500 M. This 20-fold increase should be favorable to the binding of calcium by fluoride. The increase was necessary because the bones were grown on the surface of a clot. Between the bone and the clot, diffusion is the sole means of exchanging materials.

Summary. In embryonic bones growing in tissue culture the process of calcification of cartilage can be inhibited by NaF without influencing growth. To achieve this effect the concentration of fluoride had to be 20 times greater than that which will produce inhibition of calcification in test-tube experiments. The inhibition in the present experiments is probably due to at least two things: 1st, interference with the production of phospho-pyruvate as a substrate for bone phosphatase; 2nd, some degree of binding of calcium by fluoride.

---

1. Gutman, A. B., and Gutman, E. B., PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 687.

2. Gutman, A. B., Warrick, F. B., and Gutman E. B., *Science*, 1942, v95, 461.

3. Roche, J., *Phosphatases;* Sumner, J. B., and Myrback, K., *The Enzymes*, Academic Press Inc., 1950, v1, Part 1, 473.

4. Novikoff, A. B., Potter, V. R., and LePage, G. A., *J. Biol. Chem.*, 1948, v173, 239.

5. Paff, G. H., and Eksterowicz, F. C., *Anat. Rec*, 1950, v108, 45.

6. Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, v28, 684.

Received February 25, 1952. P.S.E.B.M., 1952, v79.

Use of a Chelating Agent for Accelerating Excretion of Radio-Iron (19432)

HARRY FOREMAN,* REX L. HUFF, JOHN M. ODA, AND JOSEPH GARCIA.
(Introduced by J. H. Lawrence.)

From the Division of Medical Physics, University of California, Berkeley.

The ability of certain organic compounds to form strong non-ionic water-soluble combinations with metal ions suggests the possibility that these compounds might be useful for removing metal ions from the body. In this respect, Kety(1) has shown that use of citric acid may play a role in the removal of lead from bone by such a mechanism. Foreman and Hamilton(2) demonstrated that ethylenediamine tetra-acetic acid (EDTA) will chelate plutonium and yttrium *in vivo* and thereby greatly accelerate their excretion from the body.

This report deals with the application of this principle for hastening the excretion of radio-iron. Interest in accelerating excretion of iron is stimulated by the possibility of removing the excessive quantities of iron accumulated with hemochromatosis and transfusion hemosiderosis and for possible use as a tool for studying iron metabolism. "Fe-3 Specific"† is the compound chosen for this study. It has a very strong affinity for iron, particularly in the alkaline pH range(3). (It is used commonly for scavenging iron traces from solutions.) It was selected because it has, in addition, suitable characteristics for *in vivo* use, *i.e.*, it is relatively non-toxic, forms serum soluble chelates, is poorly catabolized, and is readily excreted through the kidney.

Methods. The effect of "Fe-3 Specific" on iron excretion was studied in a series of three experiments, using iron 59, *i.e.*, a) when iron 59 was given intravenously and "Fe-3 Specific administered intraperitoneally, b) when iron 59 was given intravenously and "Fe-3 Specific" by mouth, and c) when both iron 59 and "Fe-3 Specific" were given orally. In addition, the concentration of iron 59 in erythrocytes was studied. Adult male Slonaker

---

* Atomic Energy Commission Post-doctorate fellow, Division of Medical Sciences, National Research Council. Present address—University of California, Los Alamos Scientific Laboratory, Los Alamos, N. M.

† A commercial chelating agent manufactured by Bersworth Chemical Co., Framingham, Mass. Its structure is not revealed.

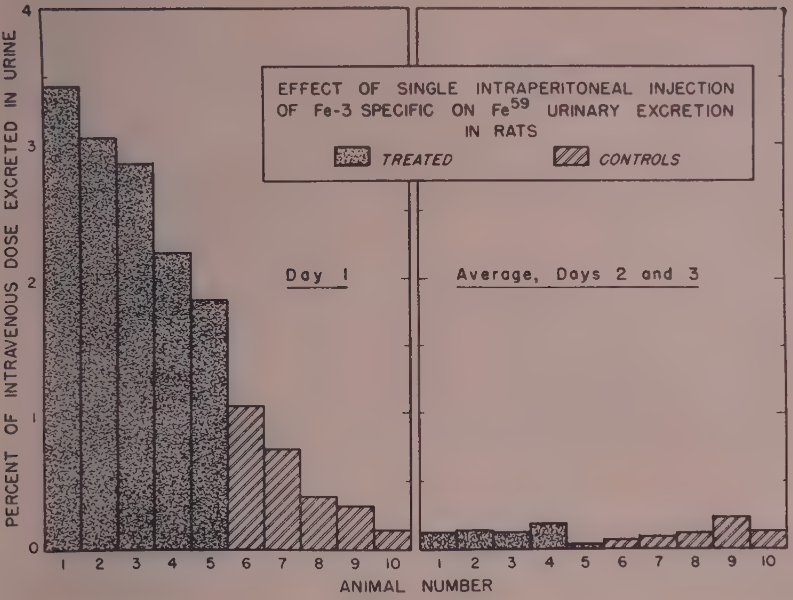


FIG. 1.

rats were used for all experiments except in Experiment 4, wherein Long-Evans rats were used. The animals were kept in metabolism cages allowing for collection of urine and feces separately. The feces samples were dry ashed at 650°C. The ash was dissolved in 10 cc of aqua regia. Two ml aliquots were withdrawn and counted directly using a scintillation counter which has been previously described (4). Urine samples were wet ashed using concentrated nitric acid and brought to 10 ml volume. Again, 2 ml aliquots were withdrawn for direct counting in the scintillation counter. *Exp. I.* Five control and 5 experimental animals were each given 2.5×10^5 c/m of iron 59, representing 5.3 μg of iron, by single intravenous tail injection ($FeCl_3$ buffered with sodium citrate). Five minutes before the iron 59 injection, the 5 experimental animals were each given 100 mg of "Fe-3 Specific" in a single intraperitoneal injection. The first excreta samples were collected 24 hours later. The results of the urinary excretion are presented in Fig. 1. Thereafter, collections were made of 2-, 3-, and 5-day periods as indicated in Fig. 2 and 3. On the 14th and 26th days following the iron 59 injection, "Fe-3 Specific" in 100 mg doses was again injected intraperitoneally into each of the experimental animals. Excretion was studied for a total of 90 days. *Exp. II.* A group of 6 rats was given a diet in which "Fe-3 Specific" was intimately mixed. The diet was prepared by mixing neutralized "Fe-3 Specific" solution with Purina Chow in such proportions that each animal received 400 mg, (*i.e.*, 20 cc of a 2.0% solution per animal daily). Five days following the initiation of the diet, the animals were each injected intravenously by tail vein with 5.3 μg of iron tagged with iron 59. The controls of Experiment I were used as controls for this experiment. Excreta collections and analyses were carried out during the next 16 days. A total of 9.6 g of "Fe-3 Specific" was fed to each animal (24 days) after which they were given the regular diet and were observed for one year. *Exp. III.* A group of 6 rats was fed in the same manner as in Experiment II. Three days after the initiation of the diet, tracer iron was added to the diet of the 6 animals receiving the "Fe-3 Specific" as well as to 6 others not receiving it. The iron intake was approximately 6.2 mg per day, and the tracer made up less than

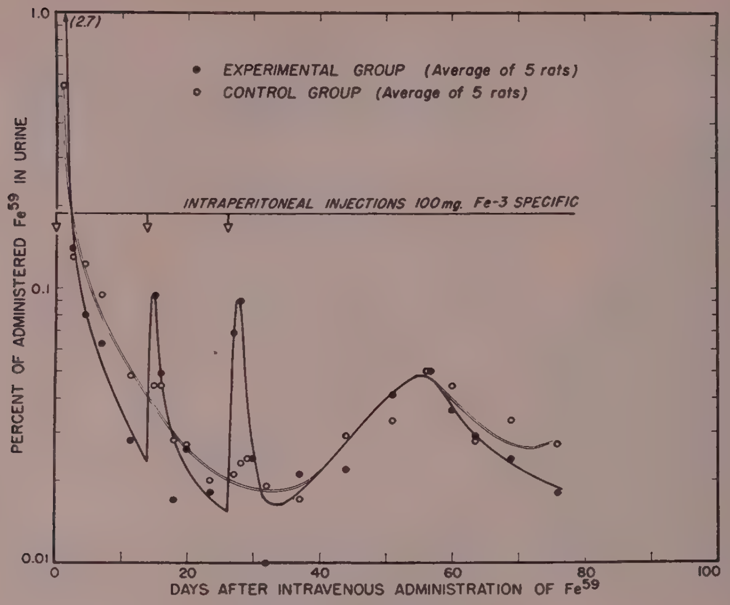


FIG. 2. Effect of single intraperitoneal injections (100 mg each) of "Fe-3 Specific" on urinary excretion rate of intravenously injected radio-iron.

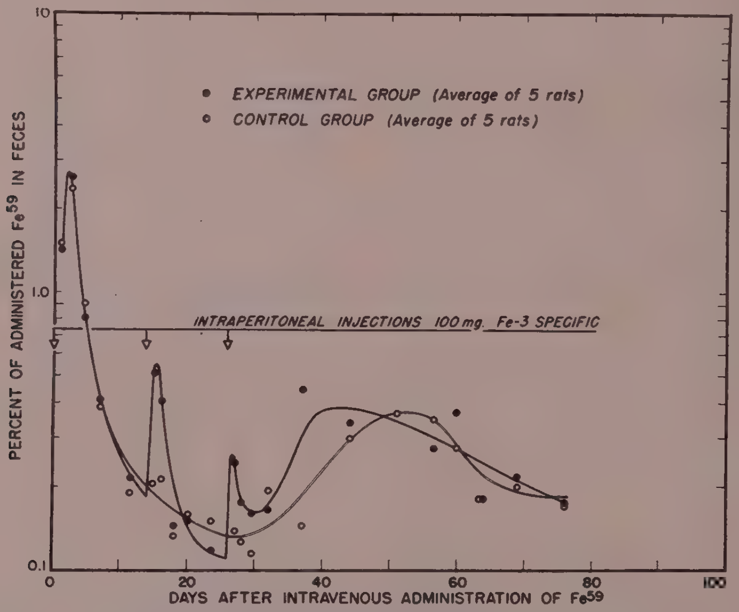


FIG. 3. Effect of single intraperitoneal injections (100 mg each) of "Fe-3 Specific" on the fecal excretion rate of intravenously injected radio-iron.

1% of this. It is not certain that the dietary iron was uniformly tagged. The iron was added as an aqueous solution of ferric chloride, intimately mixed with the feed. Excreta collections and analyses were carried out for 9 days following the addition of the tracer to the diet. *Exp. IV.* Five adult male Long-Evans rats were given a single intravenous injection of iron 59 tagged ferric chloride solution buffered with sodium citrate. Nine blood samples were taken from each of the 5 animals on the 2nd, 5th, 12th, 38th, 49th, 66th, 74th, 80th, and 87th days after the iron 59 injection. The samples were collected from an incised tail vein in dried heparinized calibrated capillary tubes. Sample size was from 58 to 80 microliters. The capillary was sealed and centrifuged at 3000 rpm. The packed cell volume was recorded and the sample analyzed for radioactivity in the apparatus described above.

Results and discussion. Fig. 1, 2, and 3 show the marked effect on the excretion of iron 59 as a result of single intraperitoneal injections of 100 mg "Fe-3 Specific". The effect is manifest in urinary excretion shortly after the injection of iron 59 and occurred each time after the "Fe-3 Specific" was administered. Approximately a 4-fold increase over the control levels was obtained. Fecal excretion was enhanced only by the injections on the 14th and 26th days and not with the original "Fe-3 Specific" injection. This, of course, indicates that the iron must undergo some transformation before it is susceptible to enhance fecal excretion by "Fe-3 Specific". The results suggest that "Fe-3 Specific" accelerates iron 59 excretion through depletion of plasma iron but since equilibrium exists between this and tissue iron, repeated prolonged administration of "Fe-3 Specific" might eventually affect tissue iron levels. Fig. 2 and 3 show an additional gradual but more sustained increase in excretion of iron 59 from about the 40th to 70th day after injection. This is thought to be related to the entrance into the excretory pool of a relatively large amount of radio-iron. Fig. 4, showing the marked drop in specific activity of the largest pool of iron 59, the circulating erythrocytes, is indicative of a source of the radio-iron for the positive excretory waves in urine and feces. The drop in specific activity in the red cell is a function of erythrocyte longevity and iron reutilization. The definite excretory maximum at 55 days affords an easy way of determining mean cell longevity without a model and without difficult if not impossible integration.

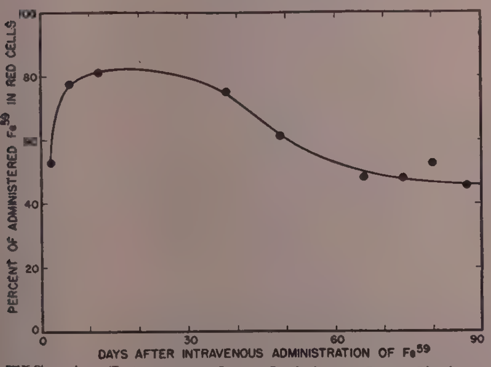


FIG. 4. Intravenously administered radio-iron present in erythrocytes as a function of time.

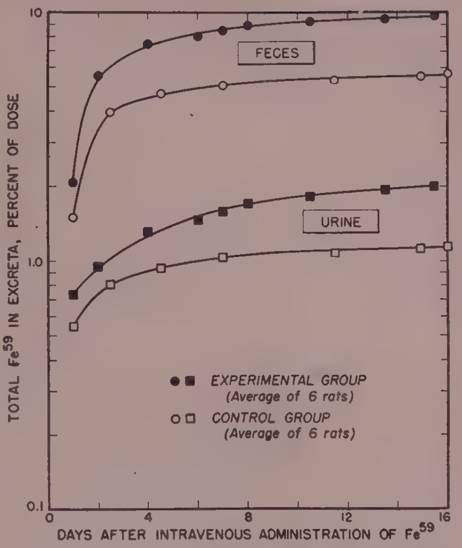


FIG. 5. Percent of a single intravenous dose of radio-iron in feces or urine of untreated rats and of rats given 400 mg "Fe-3 Specific" daily by mouth.

Exp. II, (Fig. 5), in which the animals were fed 400 mg "Fe-3 Specific" daily and were given a single intravenous injection of iron 59, demonstrated almost 10% of the tracer excreted in the feces and 2% in the urine in 16 days, in contrast to 5.7% and 1.2% in the controls. The animals of this experiment were observed for one year after receiving a total of 9.6 g of "Fe-3 Specific". No abnormalities were noted. Hematocrits remained constant during the period of "Fe-3 Specific" feeding.

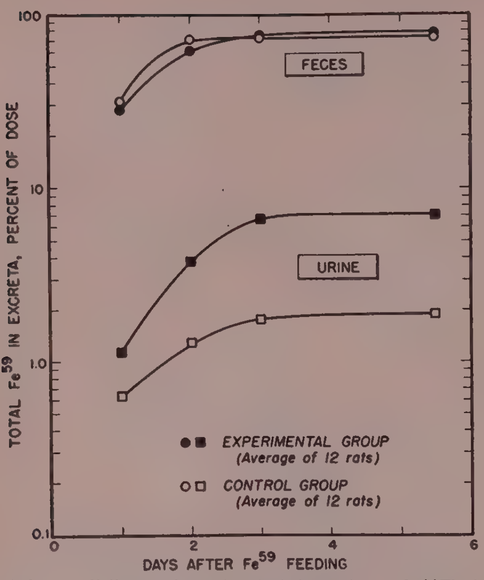


FIG. 6. Effect of oral administration of "Fe-3 Specific" (400 mg/day) on excretion of iron-59 given in a single day's diet.

In Exp. III, in which both the "Fe-3 Specific" and the iron 59 were administered in the diet, the difference in urinary excretion (Fig. 6) was as marked as in the 2 previous experiments. However, there was no great difference between fecal excretion of the control and treated animals. Approximately 7% of the administered iron was excreted in the urine of the experimental animals. Most of this occurred during the first 3 days. The urinary excretion of controls during the same period was 1.9%. The net body retention of iron 59 at the end of 7 days, was 16% and 24% in the experimental and control animals, respectively. If the diet (6.2 mg iron per day) were uniformly tagged with iron 59, then approximately 1 and 1.5 mg of iron was gained by the experimental and control animals respectively. It seems unlikely that such a great absorption could be occurring in adult rats. This would indicate that approximately 0.1 of the rat's total body iron is exchanged each day or that an even greater portion of a smaller pool is exchanged each day.

Summary. 1. Excretion of parenterally administered radio-iron is approximately 5 to 10 times as great in feces as in urine. The rate of excretion varies with the location of the tracer within the various iron pools in the body, (for example, cells, liver, reticulo-endothelial system, etc.). This is illustrated by high plasma levels of radio-iron, associated with high excretory rates in feces and urine. Fifty-five days after injection of tracer iron there is a second maximum in excretory rate which can be shown to correspond with a loss of radio-iron from the largest pool, the circulating erythrocytes. When "Fe-3 Specific" is administered intraperitoneally to animals which have been given iron 59, there is a prompt and marked increase in both the urinary and fecal excretory rates by a factor of 2 to 2.5. 2. The addition of "Fe-3 Specific" to the diet of rats causes an increase in the fecal and urinary excretion of intravenously administered radio-iron by approximately a factor of 2. 3. When labelled iron and "Fe-3 Specific" are fed simultaneously, a marked increase in urinary excretion of the tracer occurs. However, the amount found in feces is nearly the same as in controls. Thus the decrease in net retention resulted primarily because of increased urinary excretion. 4. Feeding as much as 9.6 g of "Fe-3 Specific" to animals at a rate of 400 mg per day, was not associated with any change in hematocrit nor any grossly observable abnormality in the animals.

---

1. Kety, S. S., *J. Biol. Chem.*, 1942, v142, 181.
2. Foreman, H., and Hamilton, J. C., *UCRL,* 1351, 1951.
3. The Versenes, *Tech. Bull.* 3, Bersworth Chemical Co., Framingham, Mass.
4. Anger, H. O., *UCRL,* 886, August 1950.

Received February 26, 1952. P.S.E.B.M., 1952, v79.

Further Role of Antibiotics in Aminopterin-Induced Folic Acid Deficiency in Rats.* (19433)

HARRY A. WAISMAN AND J. CRAVIOTO-M.

From the Department of Pediatrics, University of Illinois College of Medicine, Chicago, Ill.

In previous experiments(1) we have shown that the "folic acid deficiency" produced in rats by the use of the folic acid antagonist aminopterin can be overcome by the peroral or intraperitoneal administration of aureomycin. However, the "folic acid deficiency" produced in rats fed a sulfonamide drug was not corrected by aureomycin. Since preliminary experiments showed that the citrovorum factor concentrates were able to correct the deficiency produced on either type of diet, it seemed probable we were dealing with a citrovorum factor deficiency rather than a folic acid deficiency. It was suggested that aminopterin is probably more accurately a citrovorum factor antagonist than a folic acid antagonist.

Further experiments were done to provide information on the mechanism of action by which aureomycin corrected the citrovorum factor deficiency. A number of other antibiotics were studied since it was of interest to test further the hypothesis that the beneficial effect of these substances is mediated through their influence on the intestinal flora.

Experimental. Male rats of the Sprague-Dawley strain, 24 days old, were given a synthetic diet consisting of sucrose 73 parts; vitamin-free casein (Labco), 18 parts; salt mixture,[†] 4 parts; corn oil, 5 parts; thiamine, 0.5 mg; riboflavin, 0.5 mg; niacin, 1 mg; calcium pantothenate, 2 mg; pyridoxine, 0.5 mg; i-inositol, 100 mg; biotin, .002 mg; choline, 300 mg; para-amino benzoic acid, 25 mg; vitamin K, 0.5 mg. Two drops of oleum percomorphum were given by mouth twice weekly to each rat as a source of vit. A and D. Five rats were in each group and each series was repeated at least once. A total of 225 rats were used in these experiments.

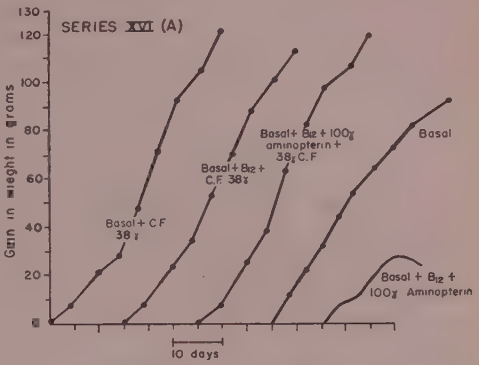


FIG. 1. Effectiveness of citrovorum factor in counteracting aminopterin.

Results. The data extend the previous findings that the addition of citrovorum factor can correct the "folic acid deficiency" induced by aminopterin, and that aureomycin is able to counteract the effect of the antagonist. Fig. 1 illustrates the weight gain obtained on a basal diet without any folic acid compounds, and the increased growth that results after citrovorum factor[‡] is added. The further addition of vit. B_{12} was without effect and it may be that adequate quantities of B_{12} occur in the purified casein so that no increased growth response could be demonstrated with additional vit. B_{12}. It will also be seen in Fig. 1 that when 100 μg of aminopterin[‡] are added to the diet containing 38 μg of citrovorum factor the antagonist is unable to produce the deficiency. From other work in this laboratory it is apparent that small amounts of citrovorum factor can counteract increasing amounts of aminopterin in a stoichiometric relationship. It should be remembered that this relationship does not exist between folic acid and the folic acid antagonists.

Fig. 2 shows the poor growth obtained with

---

[*] This work was supported in part by research grants from the National Institute of Health, Public Health Service, the American National Red Cross (Atomic Energy Commission) and Playtex Park Research Institute.

[†] Salts No. 2, Nutritional Biochemicals, Inc.

[‡] We wish to express our thanks to Lederle Laboratories, American Cyanamid Co. for supplies of aminopterin and citrovorum factor.

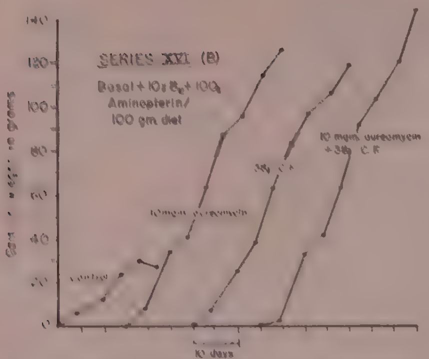


FIG. 2. Growth stimulation by aureomycin or citrovorum factor in aminopterin fed rats.

100 μg of aminopterin on the basal diet containing vitamin B_{12} and the markedly improved growth which results when 10 mg of aureomycin are included in such a diet. If 38 μg of citrovorum factor are added to the basal aminopterin diet, growth is about equal to that of the aureomycin group. This suggests that aureomycin probably does influence the bacterial flora in such a way that citrovorum factor or folic acid is provided to the host. Fig. 2 also demonstrates that when aureomycin and citrovorum factor are added together, despite the presence of aminopterin, growth is much superior to that of either aureomycin or citrovorum factor alone. It may be that when citrovorum factor is added to the diet conditions are made more favorable for the intestinal bacteria to provide still further growth factors which have not yet been elucidated.

We have found that different sources of rats can influence some of the findings in our experiments. Evidently the strain of rats, and more importantly their intestinal flora, play a part in determining what growth response the animal will have. It has been found that the rats from commercial source "A" did not provide the consistent growth response obtained with aureomycin on the aminopterin diet. More reproducible results were experienced with rats from commercial source "B". Aureomycin was able to correct the aminopterin-induced deficiency in 2 of 3 series, but in the third irregular responses were obtained. This last group of rats was obtained from the commercial source "A" and our experience with these animals indicated a variable growth response in rats receiving the same diet. When this was recognized, only rats from commercial source "B" were used.

Since the beneficial effect of aureomycin was suggestive, it was of interest to study the effect of other antibiotics on aminopterin-induced folic acid deficient diets. Fig. 3 shows that streptomycin, penicillin, terramycin and chloromycetin are unable to counteract the effect of aminopterin. In 2 of the series terramycin was ineffective although in a third series the increased weight gain was indicative of the improvement over the aminopterin-containing diet. This last observation emphasizes the variations in the sources of rats and also illustrates the importance of the previous nutritional history of the breeding females.

Discussion. These experiments have provided additional information on the action of antibiotics in the deficiency induced by a folic acid antagonist. Although we feel that the antagonist probably induced a folic acid and or citrovorum factor deficiency, no final explanation exists as to how the antibiotics are able to overcome these particular deficits. There is some evidence to support the hypothesis that the intestinal flora is providing the factors required by the host. Davis and Chow(2) gave indirect evidence when they showed that radioactive cobalt (CO^{60}) in the diet of adult rats could be incorporated in the vit. B_{12} found in the feces after aureomycin was fed. Jacob and his coworkers(3) found that intravenous aureomycin was re-excreted into the intestines and partially suppressed the fecal flora in both man and dog.

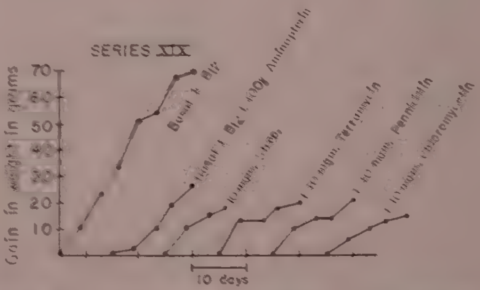


FIG. 3. Inability of 4 antibiotics to counteract effect of aminopterin in rats.

It is of interest, therefore, to speculate why the beneficial effect was not obtained with antibiotics other than aureomycin, since their antibacterial spectra are not too dissimilar. It may be that some of the antibiotics were rapidly destroyed in the intestinal tract or by body tissues and therefore could not be effective against the "deleterious" bacteria that were either consuming citrovorum factor or destroying it and therefore making it unavailable to the host. One of the greatest defects in our information at present is the fact that little is known about the synthetic abilities of a particular microorganism and less information is available on the interrelationships between the various organisms in the intestinal tract. The suppression of some bacteria and/or the resulting ability of others to flourish may account for the relative effectiveness of an antibiotic to indirectly provide the particular substance required by the host.

Summary. Streptomycin, terramycin, penicillin and chloromycetin, unlike aureomycin, were ineffective in overcoming the deficiency induced by the feeding of aminopterin to rats. Citrovorum factor is able to counteract aminopterin at low levels, indicating that the aminopterin may be a citrovorum factor antagonist as well as a folic acid antagonist. The addition of aureomycin to a diet containing citrovorum factor provides better growth than could be obtained on either supplement alone.

1. Waisman, H. A., Green, M., Cravioto, J., Ramenchik, A., and Richmond, J. B., Proc. Soc. Exp. Biol. and Med., 1951 v76, 384.

2. Davis, R. L., and Chow, B. F., Proc. Soc. Exp. Biol. and Med., 1951, v77, 218.

3. Jacob, S., Schweinburg, F. B., and Rutenburg, A. M., Proc. Soc. Exp. Biol. and Med., 1951, v78, 121.

Received March 3, 1952. P.S.E.B.M., 1952, v79.

Normal Mucoprotein Values in the Dog; Response to Distemper Vaccine and to Endocarditis.* (19434)

Robert N. Hammerstrom,[†] Forrest H. Adams, John Bussman, and
C. Walton Lillehei. (Introduced by O. H. Wangensteen.)
(With the technical assistance of Shirley Cunningham.)

From the Departments of Surgery and Pediatrics, University of Minnesota Medical School, Minneapolis, Minn.

The alterations in serum proteins in a variety of clinical conditions have received considerable attention in recent years. One field of investigation has concentrated on the mucoprotein constituents of the blood serum. It has been shown by several investigators (1-5) that an elevation in the mucoprotein concentration in the human occurs in many conditions. Winzler and coworkers(1,2) have demonstrated an increase in patients with malignancies of various types. They likewise showed that patients with pneumonia have an elevated mucoprotein level. Other infections, both of bacterial and of virus origin, are reported to be accompanied by a rise in mucoprotein values(3). It would appear that the mucoprotein component of the blood exhibits a non-specific response to body tissue injury. The only instance of reduction in the mucoprotein value clinically is that which occurs with severe renal disease(5). Recently, attention has been directed to the response of the mucoproteins in rheumatic fever(3,4,6). Adams and coworkers(6), on the basis of studies in rheumatic fever patients, have suggested that even though adrenocorticotropin therapy often produced an apparent clinical and laboratory remission, the abnormal mucoprotein elevations persisted in their patients

---

* These investigations were supported by Research Grants from the National Institutes of Health, U. S. Public Health Service, The Graduate School of the University of Minnesota, and Minnesota Heart Association.

[†] Public Health Service Research Fellow of the National Heart Institute.

until the rheumatic process became inactive.

The above mentioned studies have led to the investigation of serum mucoprotein levels in dogs under the following conditions: Group I, normal dogs; Group II, response to a distemper vaccine in normal dogs; Group III, dogs with a chronic cardiovascular stress in the form of large arteriovenous fistulas; Group IV, dogs with bacterial endocarditis produced by the intravenous injection of small numbers of bacteria following the construction of large arteriovenous shunts.

Methods of study. The analysis of the serum mucoproteins was carried out after the method of Winzler(1), using the Folin-Ciocalteau reagent, with adaptation to the Coleman Photoelectric Colorimeter. This method actually determines mucoprotein from an estimation of its tyrosine content, which has been found to bear a constant relationship to mucoprotein carbohydrate and nitrogen. *Group I. Normal dogs.* Blood for the determination of the normal mucoprotein control values was drawn, immediately upon admission to the animal colony, from each of 55 mongrel dogs, unselected as to sex or age. No animal which had any obvious evidence of infection or disease was accepted for a control determination. *Group II. Animals receiving a distemper vaccine.* The day of admission, after withdrawal of a control sample, 23 of these same animals were injected subcutaneously with 5 ml of canine Distemper Vaccine (Virogen).‡ This product is prepared from virulent canine distemper virus (20% suspension) and selected strains of the important secondary invaders—*Brucella bronchisepticus*, *Streptococcus* (pyogenic), and *Salmonella typhimurium*. It is formalin-killed, and so prepared as to preserve the full immunizing value of the virus and the bacterial antigens. They were also given an oral vermifuge (Vermiplex)§ and an insecticide was externally applied (Lexone).‖ Repeat mucoprotein determinations were made 2 days later, and thereafter at weekly intervals. *Group III. Dogs with large arteriovenous fistulas and no infection.* Mucoprotein determinations were made in 11 dogs with a severe cardiovascular stress in the form of large arteriovenous fistulas. All of these dogs had received the distemper vaccine as normal animals prior to the construction of their arteriovenous shunts. In 7 of the 11 dogs in this group the mucoprotein levels had been followed until they had returned to the normal range.¶ In the other 4 animals of this group, although actual determinations were not made, a sufficient length of time was allowed to elapse** for the mucoprotein levels to return to normal before the fistulas were made. These dogs carried either 2 lower extremity fistulas (an iliac and a femoral) or a single aorta-vena cava fistula, which were made according to technics previously described from this laboratory(7-10). The physiologic alterations in the hemodynamics and the increase in adrenal gland weights of animals with arteriovenous shunts of this size have been described(8). This group of animals had no sign of infection, and all of them had repeated negative blood cultures during the period mucoproteins were being studied. *Group IV. Dogs with large arteriovenous fistulas and bacterial endocarditis.* This fourth group of animals, in which mucoprotein determinations were made, had endocarditis produced by the standard technic employed in this laboratory(7-10). Briefly described, this method for the production of endocarditis consisted of the construction of arteriovenous fistulas of a size similar to those described for the dogs of Group III. After an interval of 4 weeks or more following construction of the shunts, small numbers (0.005 cc to 0.5 cc of a 24-hour broth culture, daily for 7 days) of Lancefield Group A†† or D‡‡ beta hemolytic streptococci were injected intravenously. These dogs then carried positive blood cultures and were proved at autopsy to have bacterial endocarditis with severe val-

---

‡ "Virogen®"—Pitman-Moore Co., Indianapolis.

§ Vermiplex—Pitman-Moore Co., Indianapolis.

‖ "Lexone"—E. I. du Pont de Nemours and Co., Wilmington, Del.

¶ Mean value and standard error of mean for these 7 dogs was 2.1 ± 0.27.

** 7 to 18 weeks.

†† Lancefield, Group A, Type XIV.

‡‡ Lancefield, Group D, "Strain I. F." See reference No. 10 for further description.

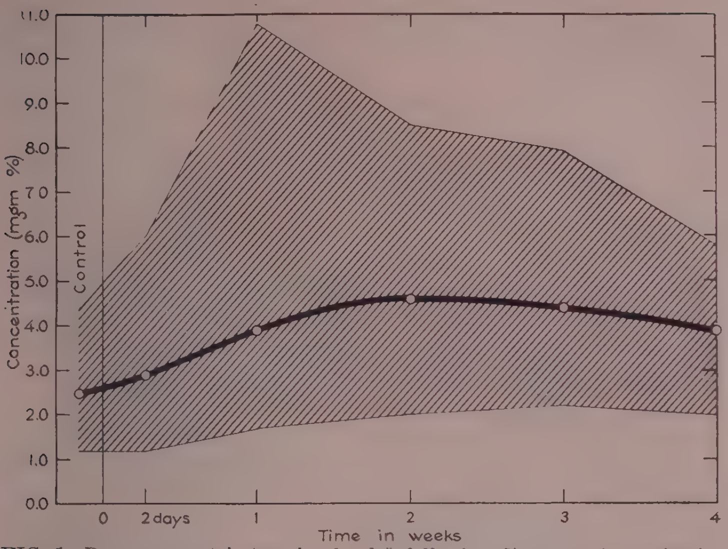


FIG. 1. Dog mucoprotein-tyrosine levels* following distemper immunization. * (Shaded area represents the range, the heavy dark line the mean values).

vular deformities due to vegetations.

Results. Group I. Normal dogs. The mucoprotein-tyrosine value of 55 normal dogs ranged from 1.2 to 4.4 mg %. The mean value was 2.5 mg % with a standard error of the mean of ±0.11 mg %. *Group II. Animals immunized against distemper.* The sequential mucoprotein-tyrosine values for this group of dogs showed a rising trend already evident 2 days after immunization, and reaching a peak level at a time 2 weeks after the immunization. Thereafter, the values show a declining trend toward normal. In the accompanying figure (Fig. 1) the heavy line represents the mean of the values for the serum mucoprotein-tyrosine determinations in the 23 dogs followed subsequent to distemper-bacterial[‡] immunization. The shaded area indicates the range of variation of these determinations. Twelve of the 23 animals were followed at weekly intervals until their mucoprotein-tyrosine values returned to normal. This occurred in the majority of animals by 7 weeks, but took as long as 18 weeks in animals which clinically developed distemper in spite of having received the vaccine. The highest (10.8 mg %) and most prolonged elevations (18 weeks) of the mucoprotein-tyrosine levels occurred in these animals. The ranges of values as shown in Fig. 1 reveal a considerable individual variation in the response to a standard vaccine. *Group III. Dogs with large arteriovenous fistulas and no infection.* The 11 dogs with large arteriovenous fistulas alone had a mean mucoprotein-tyrosine level of 3.0 mg % ±0.20 (standard error of the mean). The range in values observed varied from 1.9 to 4.0 mg %. *Group IV. Dogs with large arteriovenous fistulas and bacterial endocarditis.* The 11 dogs in this category, with large arteriovenous fistulas and a beta hemolytic streptococcic endocarditis, showed the highest mucoprotein-tyrosine levels as a group. The range for these dogs was 3.1 to 9.8 mg % with a mean of 6.0 mg % ± 0.63 (standard error of the mean).

Discussion. Statistical analyses of the results in the 4 groups of dogs studied are shown in Table I. The elevation in serum mucoprotein levels at the peak of the response to the vaccine in Group II, as compared with Group I, is significant as evidenced by a P value of <.01. The elevation in mucoprotein-tyrosine levels occurring in dogs with the cardiovascular stress produced by large arteriovenous fistulas only (Group III) as compared to the normal controls (Group I) is also significant (P <.05). The higher elevation in

TABLE I. Comparative Mucoprotein-Tyrosine Values (mg %) Statistical Analysis.

Group	No. of dogs	Range	Mean and stand. error of mean
I. Normal dogs	55	1.2–4.4	2.5 ± .11
II. ” ” 2 wk after immunization	23	2 –8.5	4.6 ± .36
III. Arteriovenous fistula dogs without infection or endocarditis	11	1.9–4	3 ± .20
IV. Arteriovenous fistula dogs with endocarditis	11	3.1–9.8	6 ± .63

mucoprotein-tyrosine levels in the animals with arteriovenous fistulas and endocarditis (Group IV) as compared with the controls (Group I) is highly significant (P value <.01). These data reveal the normal dog mucoprotein-tyrosine level, heretofore unreported, to correspond rather closely to normal human mucoprotein-tyrosine levels.

The temporary rise observed in dogs given distemper vaccine indicates that the mucoproteins may be involved in the immunological response. This observation is similar to the findings of Good *et al.*(11), who noted that a rise occurred in hyperimmunization in rabbits and rats. However, no evidence has been presented in these studies to indicate that the elevations of the serum mucoprotein levels observed in the dogs in response to distemper vaccine represents a specific immune response. This reaction of the mucoproteins may represent the non-specific response to the injection of the toxins of killed bacteria. It would be of interest to see if these observations can be clinically corroborated following routine immunization procedures in humans.

The stress of large arteriovenous fistula loads produces a small, but apparently significant increase in the dog's mucoprotein-tyrosine level. It should be noted that the stress of large arteriovenous fistulas has been shown to produce an increase in adrenal gland weight(8-10). Good and coworkers(11), in a brief report on investigations on the effect of various forms of stress on mucoproteins found an elevation to occur with chronic chilling, hyperimmunization and adrenalin injections; whereas the Arthus Phenomenon, Schwartzman Phenomenon, soft tissue destruction by cautery, adrenalectomy, and mock operation had no effect. The evidence that the serum mucoproteins reflect a stress response is, therefore, inconclusive. The highest levels of mucoproteins were observed in dogs with large arteriovenous fistulas and endocarditis, substantiating clinical observations on the mucoprotein elevation accompanying infections. The exact nature of the role of the serum mucoproteins and their ultimate significance in pathologic physiology remains to be elucidated.

Summary. 1. The normal serum mucoprotein-tyrosine level in 55 dogs has been determined. 2. The mean value for normal dogs was found to be 2.5 mg % ± 0.11, which value corresponds closely to that for normal humans. 3. Distemper vaccine produced a temporary, but significant, elevation in the mucoprotein-tyrosine levels in dogs. 4. The peak response of the serum mucoprotein-tyrosine levels to the distemper vaccine was reached in 2 weeks; and had returned to normal in the majority of animals by 7 weeks. 5. The physiologic stress of large arteriovenous fistula loads in dogs produced a statistically significant elevation in the mucoprotein-tyrosine levels. 6. The mucoprotein-tyrosine levels were found to be elevated significantly and to a much greater degree in dogs with large arteriovenous fistulas and bacterial endocarditis produced by beta hemolytic streptococci.

---

1. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.

2. Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 617.

3. Kelley, V. C., Good, R. A., and McQuarrie, I., *Pediatrics*, 1950, v5, 824.

4. Kelley, V. C., and Good, R. A., *Fed. Proc.*, 1949, v8, 359.

5. Kelley, V. C., Good, R. A. and Glick, D., *J. Clin. Invest.*, 1950, v29, 1500.

6. Adams, F. H., Kelley, V. C., Dwan, P. F., and

Glick, D., *Pediatrics*, 1951, v7, 472.

7. Lillehei, C. W., Bobb, J. R. R., and Visscher, M. B., *Surgical Forum*, W. B. Saunders Co., Philadelphia, 1951.

8. ———, PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 9.

9. ———, *Ann. Surg.*, 1950, v132, 577.

10. Lillehei, C. W., Shaffer, J. M., Spink, W. W., Bobb, J. R. R., Wargo, D., and Visscher, M. B., *Arch. Surg.*, 1951, v63, 421.

11. Good, T. A., Good, R. A., Kelley, V. C., and Glick, D., *Fed. Proc.*, 1950, v9, 178.

Received February 25, 1952. P.S.E.B.M., 1952, v79.

Neutrality Regulation Mechanisms in Rats Receiving Sulfonic Ion Exchange Resin. (19435)

EVAN W. MCCHESNEY.

From the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

Previous studies of the *in vivo* action of cation exchange resins(1,2) have shown that one of their important effects, when fed in either the H– or NH_4– form, is to increase urinary acid excretion (*i.e.*, $BH_2PO_4 + NH_3$). However, if the resin fed contains about 2.4 meq of sodium or potassium per g the urinary acid output remains the same as that of control animals receiving the same diet, without resin. From this it may be reasoned that the net fecal alkali loss which occurs as a result of ingesting the resin, on the specific diet used, must also be 2.4 meq per g. This assumes, of course, that there has been no net change in the alkali reserve of the animals during the experimental period. It constitutes one approach to the problem of determining the total *in vivo* cation uptake of the resin.

Our understanding of the effects of cation exchange resins on neutrality regulation mechanisms is largely based on studies of human subjects. The work of Irwin *et al.*(3) indicates that the ingestion of H-form resins results in a compensated metabolic acidosis. The fact that either H– or NH_4– form resins may cause acidosis is well known, and for the intended purpose, this is to some extent desirable(4). Rats receiving 10% of either carboxylic or sulfonic resin in their diets grow nearly as well as control animals on the same (resin-free) food(5,6), which indicates that they are quite able to deal with this situation over the long term. How they accomplish this is not exactly known, nor has the acidifying action of the resins been sufficiently studied quantitatively. The purpose of the work reported herein has been to study neutrality regulation during a period of resin feeding, and during a subsequent "recovery" period, in order to clarify some of these points more adequately.

Experimental procedures. Twelve female rats served as subjects for these experiments. They were kept in pairs in metabolism cages, with provision for the collection and separation of urine and feces. The former was collected in paraffin-lined beakers over thymol. Deionized water was available at all times. Regular colony diet* was provided *ad lib.* for 5 days before the test procedures began, and during the 5-day recovery period, except that no food was available for the last 16 hours of these periods. The resin period,† which intervened, was also of 5 days' duration; the animals were not fasted at its conclusion, but were immediately transferred to the colony diet. Food consumption records were kept for the resin and recovery periods, and all of the analytical data were reduced to terms of meq per 90 g of colony (or 100 g of resin-containing) diet consumed. For purposes of convenience, all urine samples were diluted to 350 cc before analysis. The analytical methods used were the same as those previously described(1,8); the data are presented in Table I.

---

* For composition, see reference (7), footnote 4. Analysis indicated that 90 g of this diet contained the following, as meq: Na, 27.4; K, 20.8; Ca, 29; Mg, 11; PO_4, 52; and Cl, 29.

† Diets of pairs 2-6 then contained 10% resin. Ammonium-form (4.6 meq NH_3 per g) and potassium-form (4.1 meq per g) were mixed to give potassium contents described in the Table.

It is evident that a partially compensated metabolic acidosis is the principal effect produced by feeding the ammonium resin: the animals respond by increasing their urinary output of acid phosphate and ammonia. There is, of course, an increase in fecal output of sodium and potassium, but very little net change in calcium and magnesium. That the latter observation is misleading is indicated by the striking decrease in fecal excretion of phosphate. The resin probably affects phosphate metabolism by binding calcium (and perhaps some magnesium), thereby releasing phosphate for absorption. The implication is, therefore, that the amount of calcium and/or magnesium actually bound by the resin is about 1.5 meq per g, not the 0.17 meq which represents the difference in experimental and control fecal outputs of these elements (per g resin ingested). This effect on phosphate metabolism has been observed previously (8,9), but perhaps has not been generally recognized. It still occurs to a slight extent in some pairs during the recovery period, but there is surprisingly little extra fecal sodium and potassium in the recovery period considering that no interval of fasting preceded it, and the digestive tracts were, therefore, not clear of resin when it began.

The decreased absorption of cations, and increased absorption of phosphate are combined to make the factor called "Net alkali loss". This loss is not entirely compensated by the increased urinary output of acid and ammonia, hence the overall effect on acid-base balance is computed as negative. There is no evidence that this effect is significantly compensated, during the recovery period, by increased acid output.

In the pairs receiving potassium resin the acid output is diminished, but by no means in exact proportion to the amount of resin potassium provided. In fact, there is even some excess acid produced in pair 5, which received more than enough potassium to offset the

TABLE I. Electrolyte Metabolism and Neutrality Regulation in Rats Receiving Sulfonic Exchange Resin.

		Urinary constituents*								Fecal constituents*						Balances		
Pair No.	mg K/10 g resin	pH	H	NH₄	Na	K	Inorg HPO₄	Cl	Excess acid†	Na	K	Ca	Mg	PO₄	Net alkali loss‡	Acid-base§	Na	K
									Resin period									
1	(control)	6.7	1.8	3.5	21.3	14.6	4.9	25.1		2	2.0	21.7	7.7	25.2	—	—	+4.1	+3.7
2	0	5.8	8.4	12.7	14.6	17.1	12.4	24.3	15.3	7.5	4.9	23.5	7.1	12.1	22.1	-6.8	+5.1	+4.5
3	108	5.9	8.5	8.3	13.4	21.9	16.4	25.9	11.5	9.6	4.6	23.9	7.5	16.5	21.3	+ .2	+4.5	+4.3
4	20	6.3	6.2	4.5	14.2	27.7	18.6	25.4	5.1	9.7	7.2	22.6	7.9	14.7	24.0	+1.4	+3.7	+5.9
5	28	6.7	3.8	4.2	14.5	33.8	19.5	25.0	2.7	8.5	7.3	21.9	7.9	14.4	21.5	+9.2	+4.7	+8.7
6	88	7.6	-0.2	1.6	12.3	37.7	15.6	24.9	-3.9	10.4	9.2	22.5	8.9	17.7	24.6	+9.5	+5.2	+11.5
									Recovery period									
1		6.9	1.7	5.5	23.5	15.1	9.5	25.2	–	3.5	3.0	23.6	9.5	29.6	—	—	+0.4	+2.7
2		7.0	1.7	7.1	25.5	15.7	10.6	27.1	1.6	3.5	2.8	24.6	9.1	27.0	3.0	-1.4	-1.6	+2.3
3		6.7	2.9	5.0	23.6	16.1	8.4	27.1	0.7	3.9	3.0	24.2	7.4	28.3	0.2	+0.5	0.1	+1.7
4		6.9	1.4	5.0	23.8	16.1	7.9	26.6	-0.8	3.7	3.2	24.1	8.5	29.8	-0.1	+0.9	-0.1	+1.1
5		6.8	2.0	6.6	24.2	17.3	9.5	26.6	1.4	3.8	2.5	22.8	7.6	24.4	2.3	-0.9	-0.6	+1.0
6		7.1	1.3	8.7	24.8	17.2	10.5	28.1	2.8	4.2	4.3	20.3	8.8	25.6	2.0	+0.8	-1.6	-0.7

* All constituents except pH are reported in terms of meq/90 g colony diet ingested. † i.e., increase of titratable acidity + ammonia over that of controls. The former represents alkali required to adjust to pH 7.5. ‡ Increase of output of all cations, + decrease of phosphate output, per 10 g resin ingested. § The calculation is: (excess urinary acid output) + (K content of resin)—(Net fecal alkali loss). ‖ Usually referred to in previous publications as Win 3000. ¶ Katonium K, trade name of Winthrop Stearns, Inc.

fecal alkali loss. It is true that a part of this potassium does not actually become available to the animals, but this is taken into account in computing the fecal alkali loss. The latter factor is a measure of the total *in vivo* cation uptake of the resin (other than for hydrogen and ammonium ions), and is quite consistent, averaging 2.4 meq per g for all pairs for the two experimental periods combined. This figure is in good agreement with that previously obtained(1), although arrived at in a somewhat different manner. The data for pairs 3 and 4 confirm the previous observation(2) that a potassium content of 1-2 meq per g resin is probably optimal.

An important feature of the results is the apparent failure of pairs 5 and 6 to compensate, during the recovery period, for the alteration in their alkali reserve which the data indicate occurred during the resin period. The positive balance is represented partly by retained potassium, as is shown by the cation balance data. Since no significant amount of compensation occurs in these pairs (or in pair2) during the recovery period, it is evident that nearly complete compensation has already taken place (as, for example, by alteration of the rate of excretion of alkali bicarbonates(10)). Also, the following observation may be of some interest.

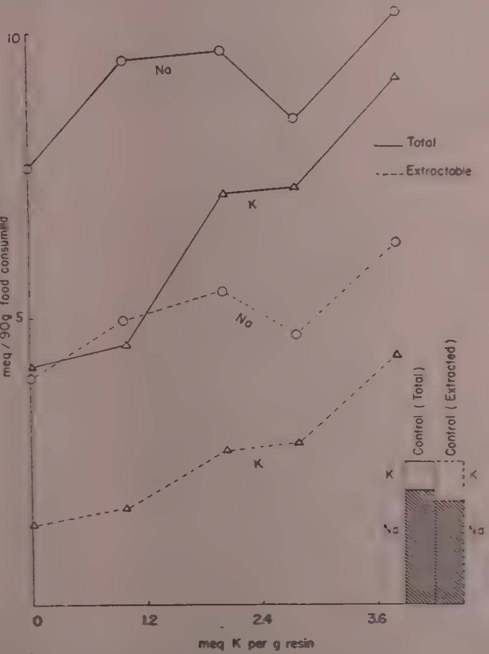


FIG. 1. Total sodium and potassium in feces, as compared to sodium and potassium extractable by water, plotted against potassium content of resin. In control animals virtually all of these elements is extractable.

Nature of resin-cation combination. It has usually been assumed that extra cations appearing in the feces as a result of resin ingestion are "bound" by it(1,2,7,8). Physical isolation of the resin from the other fecal matter would show definitely whether this is so. It is also possible(8) that hydration of the resin in the intestine leads to the retention of some electrolytes which are not directly associated with it. In order to study this point, 2% aqueous extracts of the feces of pairs 1-6 for the resin period were made,[‡] filtered, and analyzed for sodium and potassium, with the results shown in Fig. 1. It is apparent that the amounts of each of these ions in the extracts is directly related to its total concentration in that sample. About half of the sodium is extracted in each case, but the proportion of potassium extracted increases with the total amount present. In fact, in pair 2 the extractable potassium is even less than the amount found in the controls. This could mean that an exchange reaction is occurring during the extraction, with some potassium passing into the resin. This would occur if the K:Na ratio in the fecal residues exceeded that already existing in the resin. It is not clear, however, why such large proportions of these cations are present in a readily extractable form. The feces of these animals were not of a character which would suggest that any extensive water retention had occurred.[§] It also seems unlikely that the extracted ions have been released in exchange for other cations present in normal feces, except as noted above.

[‡] The finely pulverized feces were extracted with mechanical shaking for 45 minutes, then filtered. The pH of all extracts was 6.7.

[§] If the resin does cause any appreciable water retention, some of the extra cations appearing in the feces may not represent a loss of alkali, since the anions would also be retained in the intestine. This would help to explain the results in pair 2, but not in 5 and 6.

Summary. Rats receiving sulfonic acid resin respond by increasing their urinary output of acid phosphate and ammonia, but not in amounts sufficient to compensate for their fecal alkali loss. Those receiving moderate amounts of potassium resin show little change in acid-base balance, while those on high potassium resin intakes show a partially compensated alkali retention. The net fecal alkali loss amounts to 2.4 meq per g resin fed, but a large part of this represents increased phosphate absorption, presumably due to the binding of calcium and/or magnesium by the resin. There is suggestive evidence that some of the extra alkali appearing in the feces as a result of resin ingestion is not in direct combination with the resin, and this may have some bearing on the problem of neutrality regulation.

---

1. McChesney, E. W., *Am. J. Physiol.*, 1952, v168, 44.
2. ———, *J. Lab. Clin. Med.*, 1951, v38, 199.
3. Irwin, L., Berger, E. Y., Rosenberg, B., and Jackenthal, R., *J. Clin. Invest.*, 1949, v28, 1403.
4. Greenman, L., Peters, J. H., Mateer, F. M., and Danowski, T. S., *J. Clin. Invest.*, 1951, v30, 1027.
5. Flanagan, T. L., Sax, M. F., and Heming, A. E., *J. Pharm. and Exp. Therap.*, 1951, v103, 215.
6. McChesney, E. W., *Fed. Proc.*, 1952, v11, 103.
7. McChesney, E. W., and McAuliff, J. P., *Am. J. Physiol.*, 1950, v160, 264.
8. McChesney, E. W., *J. Lab. Clin. Med.*, 1952, v39, (May).
9. Chen, J. S., and Freeman, S., *J. Lab. Clin. Med.*, 1950, v35, 99.
10. Gamble, J. L., *J. Biol. Chem.*, 1922, v51, 295.

Received February 7, 1952. P.S.E.B.M., 1952, v79.

Protective Effect of Moisture on Denaturation of Human Serum Albumin By Heat (19436)

J. W. HORNIBROOK. (Introduced by M. Pittman.)
From the Federal Security Agency, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Md.

It is well known that various proteins and living organisms are more resistant to heat when dry than when in the liquid state. However, certain materials when dried to a critical level (5-10% moisture) are more unstable than the same material in solution(1). While studying this interesting fact it was noted that dialyzed albumin, though denatured by heat when thoroughly dry, was protected when containing a certain amount of moisture.

This observation is published since it may have some bearing on the discordant results obtained in studies on viability of dried bacterial cultures.

Procedure. Twenty-five per cent human serum albumin containing 1/15,000 merthiolate was dialyzed for 24 hours at 4°-6°C against several changes of distilled water. This was made up to 5% solution with distilled water and 4.5 ml quantities distributed into a number of 20 ml ampoules. To one-third of these was added 0.5 ml of water, to another third 0.5 ml of M NaCl and to the last third 0.5 ml of 5 M NaCl. This material was then dried from the frozen state in the ampoules. They were then divided in 6 groups and placed in desiccators over sulfuric acid of the following specific gravities: 1.83, 1.73, 1.61, 1.53, 1.42, and 1.31. They remained for over a week in the desiccators at room temperature under a vacuum of 1-10 mm of Hg. During this time the moisture content of the albumin in each desiccator reached a predetermined level depending upon the concentration of the sulfuric acid(2). One ampoule of each preparation was then removed from the desiccators and the moisture content determined by the U.S.P. method. The remainder of the ampoules were then sealed under air. The sealed ampoules were then autoclaved at 250°C for 2 hours, opened and reconstituted with 5 ml of distilled water. A photoelectric colorimeter (Lumetron) was used to measure the turbidities using a green (530) filter. The results were plotted as percent transmission of a water control.

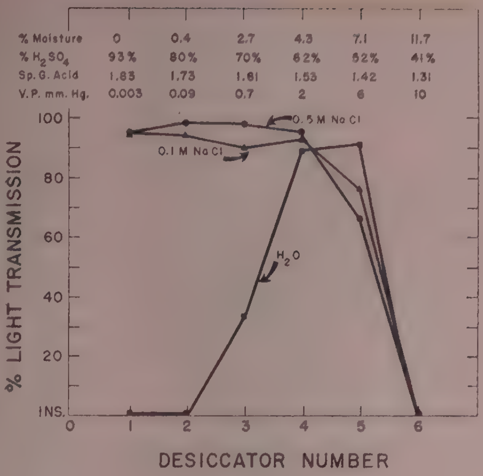


FIG. 1.

Results. Fig. 1 expresses the findings we obtained. The percent transmission roughly indicates denaturation, 100% indicating no denaturation and ins. indicating insoluble or nearly complete denaturation. The ampoules from the 6 desiccators are represented on the horizontal axis. The following additional information is recorded: Percent sulfuric acid by weight, specific gravity of the acid, water vapor pressure over the acid at room temperature, and moisture content of the albumin. The denaturation curves of the 0.1 M and 0.5 M NaCl albumin are similar to the curves which have previously been obtained for plasma and serum. These together with the albumin in water tend to become unstable at about 7% moisture and are quite unstable at 12% moisture. The albumin in water curve is most unusual in that it indicates that albumin is more stable when it contains 4% moisture than when it contains 0.4% moisture. We have found that essentially the same thing occurs when *S. marcescens* is dried in distilled water. Stability is greater when dried over 70% sulfuric acid than when dried over 93% acid.

Discussion. An explanation for the above findings is not apparent. However it is interesting to conjecture as to why this apparent anomaly should occur. It is possible that there are a number of points in the albumin molecule which have affinities for other molecules or for themselves. It is of course well known that albumin does react by binding fatty acids, certain dyes, salts, etc. Normally electrolytes or other substances would be attached to the molecule at these points. This would tend to prevent the union of these points to each other or to similar points on other albumin molecules. Such a union might result in polymerization resulting in insolubility. It seems reasonable, in the absence of such electrolytes, that water molecules might take their place, thus preventing polymerization. Calculation of the number of molecules involved makes this assumption possible. Each ampoule contains: $\frac{.214 \text{ g albumin}^*}{69000 \text{ (Mol wt albumin)}} =$ 3×10^{-6} mols albumin. Each ampoule with 0.1 M NaCl contains 5×10^{-4} mols NaCl. In the absence of salt the number of mols of water to prevent denaturation is 6.5×10^{-4}. This compares well with the figure 5×10^{-4} indicating that possibly approximately the same number of molecules of either water or salt are needed to protect against denaturation. If we divided the number of mols of water needed by the number of mols of albumin we have the figure 210 which is the number of molecules of water per molecule of albumin. Brand(3) has shown that there are 550 molecules of amino acids derived from one molecule of albumin. It is not clear what relationship these figures have to each other. However it is possible that experiments along this line may be useful in the study of the structure of albumin and other substances.

Summary. Dried dialyzed albumin is denatured by heat. One tenth M NaCl or 4 to 7% moisture prevented coagulation under the conditions of the experiment.

---

* Actual wt. of albumin in the ampoule.

1. Flosdorf, Earl W., Hull, Lewis W., and Mudd, Stuart, *J. Immunol.*, 1945, v50, 21.

2. Hornibrook, J. W., *J. Lab. and Clin. Med.*, 1949, v34, 1315.

3. Brand, Erwin. Conference on Amino Acid Analysis of Proteins. New York Academy of Sciences, Section of Physics and Chemistry October 12-13, 1945.

Received February 13, 1952. P.S.E.B.M., 1952, v79.

Growth Effects of Desoxycorticosterone and Cortisone with Special Reference to Compensatory Renal Hypertrophy.* (19437)

C. E. HALL AND O. HALL.

From the Carter Physiology Laboratory, University of Texas Medical Branch, Galveston.

Ingle(1) and others(2,3) described the inhibitory effect which cortisone exerts on the growth of normal and abnormal tissues. It has been reported that doses of cortisone which inhibit body growth, fail to affect the size of the liver, testes, pituitary or heart in rats, although causing atrophy of the adrenals, thymus and spleen(4). Desoxycorticosterone, on the other hand, is known to produce enlargement of the kidneys and heart at certain dose levels(5), and atrophy of the adrenal cortex, primarily of the glomerulosa(6).

This study compares the activity of the two hormones with respect to certain growth effects, particularly the compensatory renal hypertrophy consequent to uninephrectomy. While these studies were in progress a communication appeared which presented data indicating that cortisone augmented compensatory renal hypertrophy(7). The methods of study employed differed from those used in the present instance, and may explain the differences in data and conclusions, as compared with those presented here.

Materials and methods. Sixty-eight female rats of the Holtzman strain, weighing 52-82 g were divided into 4 groups of similar average weight. One group received 1 mg of cortisone acetate† twice daily subcutaneously; the second group received desoxycorticosterone acetate (Doca) prepared in the same vehicle, in the same amount and manner as for cortisone; the third group was restricted as to food intake so as to cause the body weight to remain static as in the cortisone-treated animals; and the fourth group served as normal controls. Each group, except that on restricted feeding, consisted of 16 animals half of which were uninephrectomized at the beginning of the experiment. The group on restricted food intake consisted of 20 animals, 8 of which were uninephrectomized. Tap water was accessible to the animals at all times and Purina Laboratory Chow was fed in limited amounts to the group on restricted food intake and *ad lib.* to all other groups. The left kidneys, removed on the first experimental day from the animals in which compensatory renal hypertrophy was to be studied, were placed in Bouin's fixative for 24 hours, washed in water and weighed after careful drying on filter paper. The experiment was terminated on the 15th day and organs to be weighed were treated in the same manner as the kidneys removed at operation.

Results. The body and organ weights, together with their standard errors, are given in Table I, with other essential data.

Growth. Growth, as judged by increment in body weight, failed to occur in rats treated with cortisone and in the untreated animals on a restricted food intake. Doca neither accelerated nor retarded growth; these animals, and the controls, almost doubled their initial weight during the experiment.

Adrenals. The adrenal glands in the animals on a restricted food intake were, in proportion to their body weight, as large as those of controls. It would appear that the degree of food restriction employed did not evoke a stress response. In agreement with previously published results a slight degree of adrenal atrophy was produced by Doca and a more marked atrophy by cortisone.

Heart. The auricles were dissected from the hearts and the ventricles weighed. These were heavier, when the weight was expressed as a function of body weight, in cortisone-treated rats than in controls. In the remaining groups the heart weight was essentially the same, being unaffected by Doca, restricted food intake or uninephrectomy.

Kidneys. The left kidneys removed at

---

* This study was supported by a grant from American Heart Association.

† The authors are indebted to Dr. Augustus Gibson of Merck and Co., Rahway, N. J. for generous supplies of cortisone, and to the Schering Corp., Bloomfield, N. J. for liberal quantities of desoxycorticosterone acetate.

TABLE I. Effect of Doca, Cortisone and Food Restriction on Growth of Body, Heart, Adrenal and Kidney.

	Data	Controls	Doca	Cortisone	Restricted diet
No. rats		8	8	8	12
†Body wt. g	Initial	66 ± 2*	67 ± 1	67 ± 3	66 ± 2
	Final	114 ± 2	125 ± 3	66 ± 4	66 ± 1
Adrenals	mg	31.1 ± 1	23.9 ± 1.1	8.2 ± 0.4	17.7 ± 0.2
	mg %	27.4 ± 1.3	19.3 ± 1.2	12.5 ± 0.8	26.9 ± 0.8
Heart	mg	290 ± 13	335 ± 14	246 ± 14	177 ± 6
	mg %	254 ± 7	268 ± 7	370 ± 15	268 ± 9
Kidneys, Left	mg	465 ± 18	599 ± 24	396 ± 16	309 ± 12
	mg %	405 ± 13	479 ± 12	598 ± 14	468 ± 12
1. Right	mg	479 ± 20	647 ± 28	412 ± 15	331 ± 8
	mg %	420 ± 15	518 ± 14	628 ± 21	501 ± 9
Renal mass	mg %	825 ± 25	997 ± 24	1226 ± 36	969 ± 19
No. rats		8	8	8	8
‡Body wt. g	Initial	67 ± 3	68 ± 2	68 ± 3	72 ± 2
	Final	107 ± 4	111 ± 3	64 ± 2	70 ± 2
Adrenals	mg	29 ± 2	25.4 ± 0.7	9 ± 0.2	19.7 ± 1.3
	mg %	27.5 ± 2.1	23 ± 0.8	14.1 ± 0.7	28.2 ± 2.1
Heart	mg	321 ± 8	297 ± 12	331 ± 17	211 ± 8
	mg %	303 ± 12	269 ± 6	513 ± 15	301 ± 15
Kidneys, Left (a)	mg	320 ± 12	306 ± 10	319 ± 11	317 ± 9
	mg %	474 ± 15	451 ± 13	457 ± 19	453 ± 11
2. Right (b)	mg	714 ± 26	848 ± 40	587 ± 17	422 ± 8
	mg %	673 ± 20	762 ± 20	915 ± 23	601 ± 12
Hypertrophy % (c)		60	47	46	20
% Renal mass intact		82	66	75	62

* Means ± Standard errors. a = at operation. b = at autopsy. $c = \frac{2-1}{1}\%$. † = Intact.
‡ = Uninephrectomized.

operation ranged, in the 4 groups, from 306 to 320 mg with an average of 315 mg. Since this was based on the weight of 32 kidneys it may be taken as representative of the weight of left kidneys of the non-operated animals at the beginning of the experiment. Therefore, the left kidneys of the intact animals averaged at the beginning 315 mg and attained a weight of 465 mg in controls; 309 mg in the restricted food group; 396 mg in cortisone-treated animals and 599 mg in Doca-treated animals. This represents an increase in actual renal mass of 47%, 0%, 25%, and 90% respectively. Kidney weight was greatest in proportion to body weight, in cortisone-treated rats, since the 25% increase in mass occurred in the absence of body growth. The degree of compensatory renal hypertrophy was calculated by subtracting the average weight of the right kidney (in mg/100 g body weight) of intact animals from the average weight of the right kidney of similarly treated uninephrectomized animals and expressing the difference in the latter as a percentage above the former. Compensatory hypertrophy was greatest in controls (60%), least in the group on restricted food intake (20%) and intermediate in the Doca (47%) and cortisone-treated rats (46%).

Discussion. In the group where body growth was held in abeyance by the restriction of food intake, kidneys of intact animals also failed to grow and the weights of the hearts and adrenals suggested that these organs grew little if at all. The proportionate suppression of growth resulted in a normal relationship between body size and the size of the visceral organs studied.

Although cortisone had the same effect on body weight as a restriction of food intake, the effect on the organ weights was quite different. The adrenals were atrophic, doubtless due to a suppression of adrenocorticotrophic hormone secretion, while the heart and kidney showed evidence of growth. In the case of uninephrectomized, cortisone-treated animals the heart weight was greater even in absolute units than in any of the other groups. This effect may have been due to a moderate degree of hypertension, since cortisone is known to produce elevations of blood pressure under certain circumstances, especially in the presence of renal insufficiency(8). However, there is no proof that any animals were hypertensive in this experiment. Although the kidneys only increased their initial mass by 25% this occurred in the absence of any corporeal growth, resulting in great hypertrophy when kidney weight was expressed as a percentage of body weight. Desoxycorticosterone acetate augmented the growth of the kidneys and produced slight adrenal atrophy, but was without any effect on cardiac weight in the dose used.

Compensatory renal hypertrophy was greatest in control animals, amounting to 60%. The remaining kidney attained a weight which was 82% that of paired kidneys of intact rats. This was reduced to 46% (75% of total renal mass of intact rats) and 47% (66% total renal mass of intact rats) by cortisone and Doca respectively. Restriction of food intake which prevented renal growth in intact rats permitted a 20% hypertrophy (62% total renal mass) in uninephrectomized animals.

Apart from variations in animal size and hormone dosage, the most obvious dissimilarity between the present experiment indicating inhibition of compensatory renal hypertrophy by cortisone, and those suggesting an augmentation, is in the method of calculating hypertrophy. In the present study this was taken to be the weight difference between the remaining right kidney of uninephrectomized rats and the right kidney of similarly treated intact rats. Gross and Meier(7) took hypertrophy to be the difference in weight between the right kidney at the end of the experiment and the left kidney removed at the beginning. This figure includes in addition to compensatory hypertrophy, normal renal growth which would have occurred in this time had the rats remained intact, and fails to consider that this might have been affected by cortisone. The use of a group on restricted food intake in the present study permitted an evaluation of the degree to which organ weight changes in cortisone-treated animals might be due to impairment of body growth.

It is apparent from these and other studies that the anti-anabolic effect of cortisone does not affect all tissues equally, and hence its effect differs from the anti-anabolic effect of restricted feeding.

Summary. 1. In a dosage of 2 mg/day cortisone completely halted body growth and produced adrenal atrophy and slight cardiac hypertrophy. Kidney size in intact cortisone-treated rats increased by 25% which, since it occurred in the absence of an increase in body weight, resulted in kidneys which were disproportionately large in relation to body size. 2. Doca in the same dosage was without effect on body weight or heart weight. Slight adrenal atrophy and moderate enlargement of the kidneys in intact animals was observed. 3. Compensatory renal hypertrophy was greatest in controls and diminished to the same extent by either Doca or cortisone. It was suppressed to the greatest extent, but not completely prevented, by a restriction of food intake to a level permitting only maintenance of a static body weight.

---

1. Ingle, D. J., *J. Clin. Endocrinol.,* 1950, v10, 1312.

2. Wooley, G. W., *Cancer Res.,* 1951, v11, 291.

3. Stoerck, H. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 798.

4. Winter, C. A., Silber, R. H., and Stoerck, H. C., *Endocrinol.,* 1950, v47, 60.

5. Selye, H., and Hall, C. E., *Am. Heart J.,* 1944, v27, 338.

6. Greep, R. O. and Deane, H. W., *Endocrinol.,* 1947, v40, 417.

7. Gross, F. and Meier, R., *Experientia,* 1951, v7, 74.

8. Perera, G. A., *Bull. N. Y. Acad. Med.,* 1952, v28, 43.

Received February 21, 1952. P.S.E.B.M., 1952, v79.

Inhibition of Pigment Formation of *Serratia marcescens* by Chloramphenicol, Aureomycin, and Terramycin. (19438)

A. J. WEIL.

From the Department of Bacteriology, Bronx Hospital, New York 56, N. Y.

The production of red pigment ("prodigiosin") by *Serratia marcescens* is subject to spontaneous variation(1,2), which presumably is genetically controlled. Moreover, pigment formation depends on certain external factors, such as temperature and the supply of magnesium and iron(3,4). Glutamic acid has just been identified by Weinberg (5) as an inhibitor of pigment production. A similar effect of chloramphenicol (chloromycetin), aureomycin, and terramycin is here described.

Methods. Four strains of prodigiosus bacilli were collected in this hospital during the summer of 1951. The fifth strain used was originally obtained from Dr. E. C. Roberts of St. Louis(7,8). The organisms were grown on 2% nutrient agar (Baltimore Biological Laboratories), pH 7,3. Temperature is not critical between 20°C and 37°C. Commercially prepared disks (Difco) were employed according to the method recently described(6) for preliminary tests with chloromycetin, aureomycin, terramycin, penicillin, streptomycin, di-hydrostreptomycin, and bacitracin. More quantitative tests were done on agar slants containing weighed amounts of antibiotic* covering a wide range of concentrations in 2-fold steps. The slants were inoculated immediately after preparation, except in some experiments, where duplicates were left at room temperature (Approximately 72°F) for 2 days in order to obtain information on the stability of the antibiotics in nutrient agar.

TABLE I. Inhibitory Effect of Chloramphenicol, Aureomycin, and Terramycin on Growth and Pigment Formation of *S. marcescens.*

	Conc. necessary to inhibit	
Antibiotic	Growth, μg/ml	Pigment formation, μg/ml
Chloramphenicol	640-1280*	10-20
Aureomycin	160- 640	10-40
Terramycin	±5000*	5-20

* One exception (see text).

Results. No inhibition of coloration or of growth was seen around disks containing penicillin, streptomycin, di-hydrostreptomycin, and bacitracin. In fact, a narrow zone of enhanced pigmentation was on occasion seen around disks with penicillin or bacitracin, which is in keeping with Weinberg's findings (5). However, wide zones of colorless growth were shown around disks with chloromycetin. The same was true for aureomycin and terramycin, though zones were somewhat narrower in width than those around chloromycetin.

The results of experiments on slants, into which antibiotics were incorporated, are summarized in Table I. Inhibition of growth by chloromycetin was obtained at concentrations between 640 and 1280 μg/ml, with the exception of one strain, which was inhibited by 40 μg. Pigment formation comparable to that of controls growing on nutrient agar without drug appeared at levels of 10 or 20 μg. With aureomycin, inhibition of growth was found to vary between 160 and 640 μg/ml, and pigment formation returned at levels between 10 and 40 μg. Terramycin inhibited growth only at the highest level tested, which was 5000 μg/ml (assuming complete solution of the amount in the agar, of which it was difficult to be sure). The only exception was the strain which had been found relatively susceptible to chloromycetin also, and which was inhibited by 160 μg/ml. Pigment formation in the presence of terramycin reappeared at levels between 5 and 20 μg. With all 3 agents, there was a trace of pink coloration visible in one or 2 tubes above the levels quoted for full pigmentation. These data refer to readings made after a period of incubation of about 18 hours. After 2 or more days, little change was seen with chloramphenicol. With terramycin, pigmentation increased in one or 2 tubes above the levels given in the preceding paragraph. With aureomycin, both partial growth and some pigmentation appeared in 2 to 4 tubes above the levels quoted. This could be correlated with the stability of the antibiotics. If growth and pigment formation on media, which had been kept for 2 days after addition of the antibiotic, was compared with that of media inoculated immediately after preparation, no noticeable difference was seen in the case of chloromycetin. With terramycin, about 50% of the inhibiting effect had disappeared. The nutrient agar containing aureomycin showed a loss of activity of over 90%.

---

* Chloromycetin, aureomycin, and terramycin were provided by the courtesy of Parke, Davis & Co., of Lederle Laboratories Division, American Cyanamid Co., and of Chas. Pfizer & Co., respectively.

If the colorless population from slants with antibiotic was plated out on nutrient agar, the ratio of red to white colonies was not significantly different from that resulting from platings of the same strain grown without drug. It appears therefore, that—excepting rare resistant mutants—pigment formation is suppressed only as long as the antibiotic is present. Also, no significant competitive advantage is gained either by the pigmented nor the colorless variant under the conditions of our experiments.

On 4 occasions, one or 2 red colonies appeared among the colorless growth at concentrations of antibiotic 2 to 8 times that generally inhibiting pigmentation. In 3 unrelated observations a few isolated colonies were noted growing well above the levels of general inhibition of growth. In these cases, growth was colorless and remained so upon propagation on media free of antibiotic. It appears that 2 kinds of mutants occurred, one showing resistance to the growth inhibiting effect of antibiotics, and one able to produce pigment in the presence of concentrations of antibiotic which usually suppress this function.

Weinberg(5) reported that penicillin and bacitracin antagonize the inhibiting effect of glutamic acid upon pigment formation. In search for similar effects, plates with discs of chloromycetin, aureomycin, and terramycin, each in concentrations of 60, 30, and 10 μg, were set up, where discs with 10 units of penicillin or 20 units of bacitracin were placed either immediately adjacent to or 7 mm distant from the first named discs. No influence upon the zones of colorless growth was noticed under these conditions.

It may be mentioned that 3 strains of heavily pigmented strains of *Pseudomonas aeruginosa* and one strain of a pink yeast were tested by the disc method against the 7 antibiotics enumerated above. No inhibition of pigment formation nor, for that matter, of growth, were observed.

These data may perhaps open an avenue of approach to the problem of the mode of action of chloramphenicol, terramycin, and aureomycin. In this connection it is of some interest that prodigiosin is a tripyrrylmethane, and as such related to the respiratory pigments (9,10).

Summary. Chloramphenicol, aureomycin, and terramycin inhibit the production of pigment by *Serratia marcescens* over a wide range of concentrations, at which the growth of the organism is not inhibited.

---

1. Reed, G. B., *J. Bact.*, 1937, v34, 255.

2. Bunting, M. J., *J. Bact.*, 1940, v40, 56, 69.

3. Bortels, H., *Biochem. Z.*, 1927, v182, 301.

4. Waring, W. S., and Werkman, C. H., *Arch. Bioch.*, 1942, v1, 425.

5. Weinberg, E. D., *J. Bact.*, 1951, v62, 785.

6. Felsen, J., and Weil, A. J., *Arch. Int. Med.*, 1951, v88, 406.

7. Weil, A. J., *J. Immunol.*, 1942, v45, 187.

8. Weil, A. J., Popken, F., and Black, J., *J. Immunol.*, 1944, v48, 355.

9. Wrede, F., and Rothhaas, A., *Z. Physiol. Chem.*, 1934, v226, 95.

10. Hubbard, R., and Rimington, C., *Biochem. J.*, 1950, v46, 220.

Received January 29, 1952. P.S.E.B.M., 1952, v79.

Effect of Vitamin B_{12} on Nucleic Acid Metabolism of the Rat.* (19439)

Irwin A. Rose† and B. S. Schweigert.

From the Division of Biochemistry and Nutrition, American Meat Institute Foundation and Department of Biochemistry, University of Chicago.

The relation between vit. B_{12} and nucleic acids has attracted considerable attention. The desoxyribosides of a number of purines and pyrimidines have been found to be active in replacing the vit. B_{12} requirement for *Lactobacillus leichmannii*(1-3), whereas the ribosides and free bases are inactive(4,5). Other studies indicated that desoxyribose-l-phosphate was not active in replacing vit. B_{12} for *L. leichmannii* when tested in the presence or absence of orotic acid, ureidosuccinate or subminimal levels of vit. B_{12}(5). The ribonucleic acid content of livers from rats fed a vit. B_{12} deficient diet was shown to be lower (decrease in basophilia) than that for rats fed a supplemented diet(6).

These experiments suggested that vit. B_{12} may function in the synthesis of nucleic acids, possibly in the synthesis of the nucleosides or their precursors. In the present report, studies on the effect of a vit. B_{12} deficiency on the amounts of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) in rat liver, and other organs, and on the rate of incorporation of N^{15} labelled glycine into the purines of RNA are presented. A preliminary report on certain phases of this study has been published(7).

Methods. Rats that had been fed a corn-soybean oil meal-iodinated casein ration with or without a crystalline vit. B_{12} supplement (24 μg/kg ration) were used in this study. The detailed composition of the rations and feeding regimen have been described previously(8,9).‡ In the initial experiment, the animals were sacrificed by decapitation after 2 weeks on experiment and the amounts of RNA and DNA in the livers were determined. The method of Schneider(10) was used in Exp. 1 and 2 for the RNA and DNA determinations with the orcinol and diphenylamine methods of analysis.§ In all cases the amounts of RNA and DNA were determined for each liver. In Exp. 2 and 3 the nucleic acid content of the spleens and kidneys was determined. In Exp. 3 a modification of the method of Ogur and Rosen(11) was used and the nucleic acids determined by their absorption at 260 mμ in the Beckman spectrophotometer. The tissue to be analyzed was homogenized in 10 volumes of cold 0.2 N $HClO_4$ and washed 2 times with this acid solution. After 2 washes with 95% ethanol the residue was suspended in 10 volumes of 1.5 N $HClO_4$ and kept at 4-6°C for 24 hours with occasional stirring. Extraction of RNA was complete and essentially no diphenylamine reacting material (DNA) was extracted in kidney or liver. Spleen could not be handled in this way since the DNA was also extracted by this treatment. The DNA was then extracted from the washed liver or kidney residue with 5 volumes of 1 N $HClO_4$ at 80° for 30 minutes. This procedure was repeated once. For the colorimetric analysis, ribose and DNA were used as the reference standards for RNA and DNA, respectively, while for the spectral analyses the extinction coefficients cited by Ogur and Rosen(11) were used as the basis for calculating the amounts of RNA and DNA in the tissues.

Results. The results (Table I) showed that the DNA content per g of liver was reduced by approximately 20% in the livers from the vit. B_{12} deficient animals as compared to the results for the supplemented group while the RNA was reduced by approximately 10% in Exp. 1. No differences were observed in the percentage of dry weight

---

* Journal Paper No. 49, American Meat Institute Foundation.

† Pre-doctoral Fellow, U. S. Public Health Service, 1951.

‡ We are indebted to H. E. Scheid, B. H. McBride, and M. Darrow for providing some of the vit. B_{12} deficient and supplemented animals.

§ We are indebted to Dr. J. M. Price for suggestions in the procedure for carrying out these determinations.

TABLE I. Effect of Vit. B_{12} Deficiency on RNA and DNA Content of Rat Liver.

Duration*	DNA (mg/g)			RNA (mg/g)		
	$-B_{12}$	$+B_{12}$	F value†	$-B_{12}$	$+B_{12}$	F value†
2 wk	2.56	3.08	13.1	6.6	7.1	
4 wk	2.37	2.99	20.8	5.1	6.9	25.6
4 wk	2.04	2.58‡	60.8	7.9	9.7‡	12.4

* 8 rats per group in Exp. 1; 7 rats in Exp. 2; and 5 rats in Exp. 3.

† F values required for significance at the 1% level were 8.68, 9.07, and 10.56 in Exp. 1, 2, and 3, respectively.

‡ Livers from rats fed supplemented diet, but with food intake restricted to that of the deficient diet were found to contain an average of 2.67 mg DNA and 9.9 mg RNA/g.

TABLE II. Effect of Dietary Treatment on Amount of RNA and DNA Per Nucleus in Rat Liver.

Dietary regimen	Body wt, g	Liver wt, g	No. nuclei per g, $\times 10^6$	mg DNA per cell, $\times 10^{-8}$	mg RNA per cell, $\times 10^{-8}$
$-B_{12}$	170	11.2	138	1.49	5.78
$+B_{12}$	240	12.4	170	1.54	5.82
$+B_{12}$ (food restricted)	172	8.5	176	1.52	5.51

or total nitrogen of the livers for the 2 groups (*i.e.*, an average of 3.70% N and 3.61% N for the deficient and supplemented group, respectively).

The results for liver in Exp. 2 and 3 substantiated those obtained in the first study (Table I). The reduction in the RNA content of the liver was greater, however, for the tests conducted for 4 weeks. These data were analyzed statistically by the analysis of variance method. The F values observed and those required for significance at the 1% level ($P = .01$) are also shown in Table I. The highly significant differences observed clearly demonstrate the constancy of the data for the animals within each group. Further, the percentage difference as well as the level of statistical significance were similar for the data obtained by the 2 methods of RNA and DNA analysis (Table I).

As in the first studies, no differences in the percentage of dry weight or nitrogen were observed in the livers for the deficient and supplemented groups. The liver weight was greater per unit of body weight, however, for the deficient animals as compared to the controls(9). The rate of gain per week for the deficient animals averaged 21 g as compared to 39 g for the supplemented rats.

In view of other observations that the amount of DNA per nucleus of the liver is constant when the test animal has been subjected to a variety of dietary stresses(12-15), it was important to determine the number of cells per g of liver for the different groups. In these tests, samples of liver from each animal in Exp. 3 (5 in each group) were homogenized (Potter-Elvehjem) and suitable aliquots stained with methyl green and the number of nuclei counted with a hemocytometer(15,16). These results (Table II) show that the number of nuclei per g was lower for the deficient group, while the food restricted group showed no reduction in the number of nuclei per g of liver. These results show that the reduction in the amounts of DNA and RNA and the number of nuclei per g of liver could be attributed to feeding the vit. B_{12} deficient diet and not to the associated reduction in the food intake. The amounts of DNA and RNA per cell were shown to be the same for the 3 groups. Therefore, the basic unit for comparison of the nucleic acid values would appear to be the cell, rather than the amounts of RNA and DNA per unit of fresh weight or dry weight of liver or liver nitrogen. It is recognized that the liver cells of the deficient animals may have been higher in fat which would dilute the other constituents of the cells. Although fat estimations were not made, gross observations and the dry weight and nitrogen determinations did not indicate that the fat content of the livers was different for the deficient and supplemented animals. One may conclude, therefore, that in a vit. B_{12} deficiency the reduction in nucleic acids per g of liver is a reflection of a decrease in the number of cells, although the amount of protein per cell was increased. These findings could be explained by the hypothesis that limiting the rate of nucleic acid synthesis by a vit. B_{12} deficiency might in turn have limited the rate of cell divisions. That vit. B_{12} de-

TABLE III. RNA and DNA Content of Spleen and Kidney from Vit. B_{12} Deficient and Supplemented Rats.

Organ*	DNA (mg/g) $-B_{12}$	DNA (mg/g) $+B_{12}$	RNA (mg/g) $-B_{12}$	RNA (mg/g) $+B_{12}$
Spleen	16.3	15.9	4.23	4.53
Kidney	8.14	8.24	2.76	2.90

* Six animals in each group. Spleens and kidneys from 2 animals in each group were pooled prior to analysis.

TABLE IV. Incorporation of N^{15} Glycine Into RNA and Protein of Rat Liver.*

Dietary treatment	Rat No.	Atom % excess N^{15} in RNA Adenine	Atom % excess N^{15} in RNA Guanine	Atom % excess N^{15} in liver protein
Basal	50	.042	.041	.073
	51	.049	.060	.083
Basal + vit. B_{12}	55	.095	.081	.100
	56	.088	.064	.099

* We are indebted to Mr. Charles McCoy, Dr. Konrad Bloch and Dr. Herbert Anker for their cooperation in these studies.

ficiency may have reduced the rate of mitosis, thus limiting nucleic acid synthesis is also a possibility. The results for the cell counts in interpreting these experiments show the usefulness of this procedure in other studies on the influence of different nutritional regimens on the nutrient content of the liver from the test animals. These measurements would not only avoid being misled in interpreting the results, but would aid in clarifying the significance of the biochemical changes observed.

Studies on the nucleic acid content of the kidney and spleen showed no differences attributable to dietary treatment. The data for the amount of DNA and RNA in these organs are shown in Table III for Exp. 2.

In an attempt to elucidate further the effect of a vit. B_{12} deficiency on the apparent rate of synthesis of nucleic acids by the rat, N^{15} labeled glycine (31.5 atom % excess) was fed to 2 vit. B_{12} deficient and 2 supplemented rats at a level of 40 mg per 100 g body weight. The glycine was thoroughly mixed with the basal ration and fed in 2 feedings; the second feeding was provided 6 hours after the first. The animals were sacrificed by decapitation 18 hours after the last feeding, the livers removed, and the adenine and guanine from the isolated RNA and DNA analyzed for N^{15}. The adenine and guanine were isolated by ion exchange chromatography(17), and the absorption maxima and minima were in good agreement with those reported by Hotchkiss (18). The residual protein remaining after removal of RNA and DNA(10) was also retained for isotopic analysis. The pertinent data are presented in Table IV. No significant incorporation of N^{15} occurred in the DNA in these studies.

Although the results show that the rate of synthesis of the purines of RNA from N^{15} labeled glycine for the supplemented animals was approximately twice that for deficient animals, an increase in incorporation of glycine into liver protein also occurred for the supplemented animals. With the protein data in Table IV as a base line of comparison, the resultant small differences in the rate of incorporation of glycine into the RNA purines are of doubtful significance with respect to the effect of vit. B_{12}. This conclusion seems warranted also in view of the difficulties that are associated with the use of animals of different body weights and of different liver weight to body weight ratios. A subsequent experiment was conducted and the difference in the rate of incorporation of N^{15} glycine in the RNA purines of the livers from vit. B_{12} deficient rats as compared to supplemented rats was not any greater than in the first experiment.

It may be concluded, therefore, that although differences in the RNA and DNA content of the livers, and in the rates of synthesis of RNA purines could be shown that were attributable to the vit. B_{12} deficiency, these differences could be largely explained by taking into consideration the number of nuclei per g of liver of the rats in the 2 groups.

Summary. 1. The amounts of desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the livers of rats fed a vit. B_{12} deficient diet were significantly lower per g of fresh or of dry liver or of liver nitrogen than those for the supplemented rats. No difference was observed, however, in the amounts of RNA and DNA per cell for the deficient or supplemented rats. The amount of nucleic acids per g of spleen or kidney was the same

for the deficient and supplemented group. 2. Other studies indicated that the rates of incorporation of N^{15} glycine into the purines of RNA were lower for the deficient animals. 3. These studies suggest that in a vit. B_{12} deficiency the reduced rate of nucleic acid synthesis in the liver reduced the rate of cell divisions.

---

1. Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, 1949, v177, 993.

2. Shive, W., Ravel, J. M., and Harding, W. M., *J. Biol. Chem.*, 1948, v176, 991.

3. Kitay, E., McNutt, W. S., and Snell, E. E., *J. Bact.*, 1950, v59, 727.

4. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *J. Biol. Chem.* 1950, v184, 211.

5. Schweigert, B. S., Rose, I. A., and Friedkin, M., unpublished data, 1950.

6. Stern, J. R., Taylor, M. W., and Russell, W. C, PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 551.

7. Rose, I. A., and Schweigert, B. S., *Abstracts, Am. Chem. Soc.*, Sept., 1950, p55c.

8. Scheid, H. E., McBride, B. H., and Schweigert, B. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 236.

9. Scheid, H. E., Andrews, M. M., and Schweigert, B. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v78, 558.

10. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.

11. Ogur, M., and Rosen, G., *Arch. Biochem.*, 1950, v25, 262.

12. Davidson, J. N., *Biochem. J.*, 1945, v39, lix.

13. Davidson, J. N., and Leslie, I., *Cancer Res.*, 1950, v10, 587.

14. Campbell, R. M., and Kosterlitz, H. W., *J. Physiol.*, 1947, v106, 12P.

15. Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Res.*, 1950, v10, 18.

16. Price, J. M., and Laird, A. K., *Cancer Res.*, 1950, v10, 650.

17. Cohn, W. E., *Science*, 1949, v109, 377.

18. Hotchkiss, R. D., *J. Biol. Chem.*, 1948, v175, 315.

Received December 11, 1951. P.S.E.B.M., 1952, v79.

Coccidioides immitis Spherule Antigen in a Complement Fixation Test For Experimental Coccidioidomycosis. (19440)

RALPH A. VOGEL AND NORMAN F. CONANT.

From the Department of Bacteriology, Duke University School of Medicine

Prior to 1937 few investigators were successful in demonstrating an immune response to *Coccidioides immitis* in patients with coccidioidal granuloma. Cooke(1) obtained a positive precipitin test with serum from a patient using antigens prepared from dried powdered cultures, and extracts of pus-containing spherules. Davis(2) obtained a positive complement fixation test with serum from a patient using an antigen prepared from a macerated mycelial culture. Chipman and Templeton(3), however, were the first to use successfully a broth filtrate of *Coccidioides immitis* as an antigen in a complement fixation test. This type of antigen has since proven effective and is now widely used. C. E. Smith and associates(4) have used the broth filtrate, coccidioidin, as antigen in a precipitin test and complement fixation test for diagnosis and prognosis in coccidioidomycosis.

In a preliminary report(5) it was shown that the spherules or tissue phase of *Coccidioides immitis* could be obtained in abundance by inoculation of the yolk sac of chick embryos with arthrospores of *Coccidioides immitis*. The use of the spherules as antigen in the complement fixation test is presented in this paper.

Methods. The yolk sacs of 10-day-old embryonated hens' eggs were inoculated with the arthrospores of *Coccidioides immitis* as described in a previous report(5). Up to 0.5 ml of egg yolk from the initially inoculated embryos was passed to the yolk sac of a second series of 10-day-old embryos. After 9 days incubation the yolk of the second transfer eggs was harvested and treated with 4 parts of 10% NaCl, and allowed to stand overnight in the refrigerator. The suspension of yolk and spherules was centrifuged at 3000 r.p.m. for ½ hour. The fat which collected at the surface during centrifugation was decanted along with the soluble egg protein in the supernate. The sediment containing the spherule material was washed several times with physiological saline and finally suspended in veronal buffer solution, pH 7.3-7.4. A 1:50 concentration of spherules by volume was obtained from 10 eggs. Equal aliquots of spherule suspensions were treated with a) 5% formalin for 12 hours, and b) heat-killed in a 60°C water bath for 30 minutes. Excess formalin in the spherule material was neutralized with sodium metabisulfite as recommended by Cox.* Before use in the complement fixation test the antigens were diluted and ground for ½ hour in a small Ten Broeck glass tissue grinder. *Coccidioidin. Coccidioides immitis,* strain 2023, was grown in a synthetic medium(6) for 4 months, the fluid content filtered through a Seitz filter and the filtrate stored immediately at —20°C without preservative. This filtrate, a clear amber colored fluid, was found to be a suitable antigen for the complement fixation test in an optimal dilution of 1:16. *Sera from infected animals.* Seven white rabbits, each weighing approximately 2 kg, were inoculated intravenously with 1 ml of a 1:1000 concentration (by volume) of Coccidioides arthrospores. Two weeks later the rabbits were bled to death and their sera was stored in small amounts at —20°C without preservative. *Sera from hyperimmunized animals.* Six white rabbits each weighing approximately 2 kg, were inoculated intravenously with 1 ml of a 1:50 concentration of spherule antigen on 4 consecutive weeks and were bled to death 7 days after the last inoculation. The sera were stored in small amounts at —20°C without preservative. At no time during the vaccination period did the animals show any reaction to the injected material. *Complement fixation test.* Three 50% units of guinea pig complement(7) were used in the test. All reagents were diluted in veronal buffer, pH 7.3-7.4, and used in a volume of 0.3 ml. A dilution of 1:3 serum, inactivated at 60°C for 30 minutes, served as unity for subsequent 2-fold, serial dilutions. After 16-18 hours refrigeration at 3-6°C, 0.6 ml of optimally sensitized sheep cells were added and the tubes placed in a water bath at 37°C for 30 minutes. All tubes were centrifuged at 2500 r.p.m. and the degree of hemolysis was compared with color standards prepared to represent 0, 25, 50, 75, and 100% hemolysis interpreted as 4+, 3+, 2+, 1+ and no fixation. Tubes showing 50% hemolysis or less were considered positive. *Absorption of infected and hyperimmune sera.* The absorption technic outlined by Krumweide *et al.*(8) was used to absorb non-inactivated sera from infected and hyperimmunized rabbits. The egg yolk antigen used for absorption was prepared by suspending the yolk from one 18-day-old incubated egg in distilled water. Three ml aliquots of a 1:3 dilution of serum from a hyperimmunized rabbit and an infected rabbit were added to a) the washed sediment of 4 ml of centrifuged egg yolk antigen, and b) the centrifuged sediment of 5 ml of stock spherule vaccine. The sera were placed in a water bath at 37°C for 5 hours and then refrigerated for 15 hours. The tubes were shaken often during the incubation periods. It was necessary to repeat the test twice to obtain complete absorption of the sera.

Results. The antigenic titer of the heat killed spherule antigen was taken as that dilution of antigen beyond which further increase failed to enhance complement fixation. The antigen, despite its concentrated appearance, was not anticomplementary either undiluted or diluted 1:2. The optimal antigen dilution for testing in rabbit sera was 1:4. Formalized antigen failed to fix complement in these experiments. The results of complement fixation tests with sera from 7 rabbits inoculated with *Coccidioides* arthrospores using coccidioidin, heated spherule antigen and antigen prepared from egg yolk are presented in Table I. The titers obtained with the spherule antigen were slightly higher in some instances than those to coccioidin. The egg yolk antigen failed to fix complement in these sera, while all three antigens failed to react in the sera from 6 normal rabbits tested independently.

---

* Personal communication.

TABLE I. Reactions in Complement Fixation Test Employing Heated Spherule Antigen, Coccidioidin, and an Egg Yolk Antigen and Serum from Rabbits Infected with *Coccidioides immitis.*

Antigens	Rabbit	Serum dilutions 1-1	1-2	1-4	1-8	1-16	1-32	1-64	Controls Antigen	Serum
1	28	4+	4+	4+	2+	—	—	—	—	—
2		4+	4+	4+	4+	2+			—	
3		—	—	—	—	—	—	—	—	
1	23	4+	4+	4+	3+	—	—	—	—	—
2		4+	4+	4+	3+	—	—	—	—	
3		—	—	—	—	—	—	—	—	
1	27	4+	4+	4+	4+	4+	2+	—	—	—
2		4+	4+	4+	4+	4+	2+	—	—	
3		—	—	—	—	—	—	—	—	
1	11	4+	4+	4+	3+	—	—	—	—	—
2		4+	4+	4+	4+	3+	1+	—	—	
3		—	—	—	—	—	—	—	—	
1	12	4+	4+	4+	2+	—	—	—	—	—
2		4+	4+	4+	4+	4+	3+	2+	—	
3		—	—	—	—	—	—	—	—	
1	14	4+	4+	4+	3+	2+	—	—	—	—
2		4+	4+	4+	4+	3+	1+	—	—	
3		—	—	—	—	—	—	—	—	
1	31	4+	4+	4+	4+	2+	—	—	—	—
2		4+	4+	4+	4+	2+	1+	—	—	
3		—	—	—	—	—	—	—	—	
1	Normal*	—	—	—	—	—	—	—	—	—
2		—	—	—	—	—	—	—	—	
3		—	—	—	—	—	—	—	—	

* Represents the reaction of 6 rabbit sera tested independently.
1—Coccidioidin. 2—Spherule. 3—Egg yolk.

Hyperimmune sera. The sera from 6 rabbits immunized with heat killed spherule antigen gave high titers to both spherule antigen and egg yolk antigen revealing the complicating factor of a concurrent stimulation of egg yolk antibodies during the immunization with this antigen (Table II). In only a few instances was it possible to demonstrate a titer to coccidioidin and, when evident, always in a low titer.

Absorption studies. Egg yolk antigen completely removed egg yolk antibody in the serum from immunized rabbit No. 6 (Table II). After absorption, this serum showed a reduction in titer to 1:4 with spherule antigen while the titer to coccidioidin was unchanged. Absorption of this serum with spherule antigen completely removed antibodies to spherule antigen, egg yolk and coccidioidin. Absorption of a serum from an infected rabbit with egg yolk antigen and spherule antigen failed to lower the titer to spherule antigen or coccidioidin.

Discussion. The spherule antigen prepared from the yolk of embryonated eggs inoculated with the arthrospores of *Coccidioides immitis* appears to have the ability to fix complement in the sera of rabbits infected with *Coccidioides immitis* and in the sera of rabbits hyperimmunized with the spherule antigen. The spherule antigen is also capable of stimulating in low titer, antibodies to coccidioidin, the broth filtrate, which is used in the standard complement fixation test for coccidioidomycosis. A preliminary testing in human serum with both antigens was made. The spherule antigen failed to react in the serum of a patient with coccidioidal granuloma showing a titer of 1:32 to coccidioidin.

Summary. 1. A method is described for the preparation of a serologically active antigen from egg yolk material containing spherules. 2. "Spherule antigen" used in a complement fixation test reacted with sera from animals infected with *Coccidioides* and animals hyperimmunized with the antigen. 3. The antigen

TABLE II. Reaction in Complement Fixation Test Employing Heated Spherule Antigen, Coccidioidin, and an Egg Yolk Antigen and the Sera from Rabbits Immunized Intravenously with Spherule Vaccine.

		Serum dilutions								Controls	
Antigens	Rabbit	1-1	1-2	1-4	1-8	1-16	1-32	1-64	1-128	Antigen	Serum
1	5	—	—	—	—	—	—	—	—	—	—
2		3+	3+	3+	3+	3+	3+	1+	—	—	
3		2+	3+	3+	2+	—	—	—	—	—	
1	1	—	—	—	—	—	—	—	—	—	—
2		4+	4+	4+	2+	—	—	—	—	—	
3		4+	3+	—+	—	—	—	—	—	—	
1	6	4+	2+	—	—	—	—	—	—	—	—
2		4+	4+	4+	4+	3+	1+	—	—	—	
3		4+	4+	4+	2+	2+	1+	—	—	—	
1	2*	2+	—	—	—	—	—	—	—	—	—
2		4+	4+	4+	4+	3+	2+	—	—	—	
1	4*	1+	—	—	—	—	—	—	—	—	—
2		4+	4+	4+	4+	4+	3+	2+	—	—	
1	3	1+	—	—	—	—	—	—	—	—	—
2		4+	4+	4+	4+	3+	3+	—	—	—	
3		4+	4+	4+	4+	2+	—	—	—	—	

4—Complete fixation (no hemolysis); –, no fixation (complete hemolysis).
* Sera from these rabbits were not tested with egg yolk antigen.
1—Coccidioidin. 2—Spherule. 3—Egg yolk.

was found to stimulate low titers to coccidioidin in hyperimmunized animals. 4. The "Spherule antigen" completely absorbed antibodies to the homologous antigen and coccidioidin from the serum of a hyperimmunized rabbit, but failed to absorb antibodies from the serum of a rabbit infected with *Coccidioides immitis.*

---

1. Cooke, J. V., *Arch. Int. Med.,* 1915, v15, 479.
2. Davis, D. S., *Arch. Dermat. and Syph.,* 1924, v9, 577.
3. Chipman, E. D., and Templeton, A. J., *Arch. Dermat. and Syph.,* 1930, v21, 259.
4. Smith, C. E., Saito, M. T., Beard, R. R., Kepp, R. M., Clark, R. W., and Eddie, B. U., *Am. J. Hyg.,* 1950, v52, 1.
5. Vogel, R. A., and Conant, N. F., *J. Bact.,* 1952, in press.
6. Smith, C. E., Whiting, E. G., Baker, R. R., and Saito, M. T., *Rev. Tuberc.,* 1948, v57, 330.
7. Kent, J. F., and Rein, C. R., *Science,* 1948, v103, 598.
8. Krumweide, C., Cooper, G., and Provost, D. J., *J. Immunol.,* 1925, v10, 55.

Received March 3, 1952. P.S.E.B.M., 1952, v79.

Identification of Ferritin in Blood of Dogs Subjected to Radiation from an Atomic Detonation.* (19441)

THOMAS J. HALEY, M. R. ANDEM, RICHARD F. RILEY, AND I. WILLIAMS
From the Atomic Energy Project, School of Medicine, University of California at Los Angeles.

Direct observations on the mesoappendix capillary bed in the roentgen ray irradiated rat demonstrated that a pronounced peripheral vasodepression occurred during the first post-irradiation week. Further investigation showed that this effect was not due to a direct effect of the radiation on the capillaries themselves but was the result of the appearance of a vasodepressor agent in the blood. It was shown that this vasodepressor was liberated by the liver and had all of the characteristics of ferritin(1).

---

* This article is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

The present investigation furnishes presumptive evidence for the presence of ferritin in the blood of dogs subjected to the radiation from an atomic detonation.

Experimental. Male CFW strain rats weighing 125-200 g (average 150 g) were anesthetized with sodium pentobarbital (45 mg kg intraperitoneally) and prepared for observation of the mesoappendix capillary bed by the method of Chambers and Zweifach(2). Plasma samples were obtained from dogs subjected to a lethal dose of irradiation. The dog plasma or its dilutions with physiological saline were administered intravenously to standardized normal rats in a volume of 0.5 ml. All samples were designated by code to avoid cognition by the assayist and remove personal bias from the observations. Freshly prepared solutions of epinephrine in doses of 0.1 ml were applied topically to the mesoappendix preparation and a selected vessel studied for changes in sensitivity of its precapillary sphincters and in vasomotion. The epinephrine concentrations used for screening sensitivity tests were 1:1 x 10^7, 1:8 x 10^6, 1:6 x 10^6, 1:4 x 10^6, 1:2 x 10^6, 1:1 x 10^6, 1:1 x 10^5, and 1:1 x 10^4; while concentrations of 1:3 x 10^6, 1:1 x 10^6, 1:9 x 10^5, 1:6 x 10^5, 1:3 x 10^5, 1:1 x 10^5, 1:9 x 10^4, 1:6 x 10^4, 1:3 x 10^4, and 1:1 x 10^4 were used in titering vasodepressor activity of the plasma. The time interval between epinephrine applications was 10 minutes and the observation period was 5 minutes. All positive responses were checked to eliminate the possibility of errors in observations, and a number of samples were recoded and rerun to check the general reliability of the observations. Serial dilutions in physiological saline of a number of active samples were evaluated in order to obtain some information on the relative amounts of vasodepressor material present in these active samples. Selected samples of dog plasma whose vasodepressor activity had been previously determined to be high were aerobically incubated with minced rat liver or kidney in a Dubnoff shaking apparatus at 37.5°C and re-evaluated for vasodepressor activity. A highly active sample was dialyzed overnight in the refrigerator against physiological saline and the dialyzate as well as the sample was evaluated for vasodepressor activity. Crystalline dog ferritin was prepared from dog liver by the method of Granick(3). Rabbit anti-dog ferritin serum was prepared by injecting intravenously or subcutaneously well-dialyzed recrystallized dog ferritin into New Zealand rabbits. Precipitin reactions against dog ferritin showed that this antiserum was highly active. Known amounts of dog ferritin or active samples were incubated with various amounts of this antiserum and assayed for vasodepressor activity. Incubation was carried out at 43°C for various time intervals and in all cases for at least one hour before testing.

Results. Observations on the capillary effects of thirteen control plasma samples obtained from 8 dogs prior to irradiation showed only one plasma sample had vasodepressor activity. However, after irradiation a pronounced vasodepressor activity of 5 to 6 days' duration was observed starting irregularly on the first, second or third post-irradiation in 6 of the dogs' plasma samples. The other 2 dogs' plasma samples showed the presence of a vasodepressor material but did not have the same overall pattern of appearance. The loss of vasodepressor activity occurred on the sixth, seventh or eighth post-irradiation day when a normal epinephrine sensitivity and vasomotion response was obtained in the standardized test animal. These results are summarized in Table I.

The irregularity of appearance of the vaso-

TABLE I. Sequence of Appearance of Ferritin in Dog Plasma After Atomic Irradiation.

Dog	Control		Post-irradiation day							
No.	1	2	1	2	3	4	5	6	7	8
51	—	—	—	I	I	I	A	I	A	A
[illegible]	I	I	I	I	I	A	I	[illegible]	—	—
[illegible]		I	A	A	A	A	A	[illegible]	[illegible]	
[illegible]	I	I	A	A	A	A	A	[illegible]	[illegible]	I
[illegible]	I	I	A	A	I	I	—	A	—	—
[illegible]	I	I	I	A	A	A	A	A	[illegible]	—
[illegible]	I	I	I	I	A	A	A	A	A	—
[illegible]	I	A	A	A	A	I	A	[illegible]	A	I

I—Inactive, no ferritin present.
A—Active, ferritin present.
* Results equivocal.

TABLE II. Titration of Ferritin (VDM) in Plasma of Irradiated Dogs.

Post irradiation day*	Dog 19		Dog B-83		Dog A-90	
	No. vasodepressor† doses/ml plasma	Dog Ferritin N equivalents, γ/ml	No. vasodepressor doses/ml plasma	Dog Ferritin N equivalents, γ/ml	No. vasodepressor doses/ml plasma	Dog Ferritin N equivalents, γ/ml
1	>15<30	>.015 <.50	>50<500	>.05 <.50	>300	.30
2	>15	.015	>10<50	>.01 <.05	>50<300	>.05 <.30
3	>15	.015	<10	.01	>15	.01
4	0	0	~2	.002	>15	.015
5	0	0	~2	.002	>15	.015

* Time is marked from the first day of appearance of vasodepressor activity.
† One vasodepressor dose = 0.001 γ dog ferritin N/ml.

depressor material (Table I) prompted an evaluation of the amount liberated and the duration of its presence in the plasma. Table II gives the titers on the plasma of three dogs. These evaluations are based on the fact that 0.001 γ N ml is the smallest amount of ferritin which can be consistently detected by the mesoappendix text(1,4). The results presented in Table II indicate that initially a large amount of VDM is released after irradiation and the amount is reduced as the organism begins to recover from the initial damage produced by the irradiation.

Inasmuch as similar radiation studies with roentgen ray irradiated rats indicated that the vasodepressor material appearing in the blood after irradiation was presumably ferritin(1), we applied several of the tests for ferritin proposed by Mazur *et al.*(5). Active plasma samples from dogs on the first, second and third post-irradiation days (dogs 19, B-83 and A-90 respectively) were incubated aerobically for 3 hours at 37.5°C with minced kidney and with minced liver. These samples were inactivated by liver but not by kidney. When 3 ml of active dog plasma containing at least 40 vasodepressor doses per ml was dialyzed overnight in the refrigerator against 13 ml of physiological saline, there was no decrease in the vasodepressor activity of the sample, nor any appearance of activity in the dialysate. Injection of crystalline dog ferritin or vasodepressive dog plasma into previously standardized normal rats gave a vasodepression which could be entirely prevented by pre-injection or entirely counteracted by post-injection of 1 ml of rabbit antidog ferritin serum. These highly successful *in vivo* inactivations were in contrast to numerous less successful attempts at *in vitro* inactivation in which various dilutions of the antiserum were incubated with samples of active plasma. Normal rabbit serum did not inactivate either the ferritin or the active plasma.

Discussion. The following text table gives the criteria established by Mazur, *et al.*(5) for the identification of ferritin and shows the similarity between ferritin and the vasodepressor material found in the blood plasma of dogs subjected to radiation from an atomic detonation.

Comparison of Radiation VDM and Ferritin

Criteria	*Ferritin and Dog Plasma VDM*
Effect on capillary bed	Decreased epinephrine sensitivity
Effect on vasomotion	Greatly decreased
Dialysis	Does not dialyze
Aerobic incubation	
Liver	Inactivates
Kidney	Does not inactivate
Effect of antiserum	Inactivates

The completely parallel findings taken together with the fact that no other compound has been shown to produce such profound peripheral vascular effects at such a low dose (6) provides presumptive evidence for the identity of the VDM in the blood of dogs subjected to radiation from an atomic detonation as ferritin.

Summary. Studies on blood plasma samples obtained from eight dogs subjected to a lethal dose of radiation from an atomic detonation demonstrated that a material was present which, when administered intravenously, decreased the rate of vasomotion and decreased the epinephrine sensitivity of the mesoappendix capillary bed of the normal rat. After its appearance in the blood, the concentration of this vasodepressor material decreased as a function of time. This VDM has been shown to fit the criteria established for ferritin.

The authors wish to express their sincere thanks to Dr. R. L. Libby of the School of Medicine, University of California at Los Angeles, for collecting and supplying us with the plasma samples.

---

1. Haley, T. J., Riley, R. F., Williams, I., and Andem, M. R., *Am. J. Physiol.*, in press.
2. Chambers, R. and Zweifach, B. W., *Am. J. Anat.*, 1944, v75, 173.
3. Granick, S., *J. Biol. Chem.*, 1943, v149, 157.
4. Mazur, A. and Shorr, E., *J. Biol. Chem.*, 1948, v176, 771.
5. Mazur, A., Litt, I., and Shorr, E. P., *J. Biol. Chem.*, 1950, v187, 473, 485, 497.
6. Zweifach, B. W., Chambers, R., Lee, R. E., and Hyman, C., *Ann. N. Y. Acad. Sci.*, 1948, v49, 553.

Received March 10, 1952. P.S.E.B.M., 1952, v79.

Portal Venipuncture. A Percutaneous, Trans-Hepatic Approach. (19442)

HOWARD R. BIERMAN, HOWARD L. STEINBACH, LAURENS P. WHITE, AND KEITH H. KELLY.

From the National Cancer Institute, National Institutes of Health, Public Health Service, and the Divisions of Medicine and Radiology, University of California School of Medicine, San Francisco.

Many clinicians and physiologists have wanted a simple method of access to the portal venous system. If direct access were possible, not only could studies be made on portal venous blood, but also the portal venous system could be demonstrated by injecting a contrast medium and making roentgenograms. Hitherto, samples of blood from the portal vein have been obtained during laparotomy and, in a few instances, polythene tubing has been tied into the portal vein and brought out through the wound for sampling at a later time. In a few patients with hepatic vascular obstruction, small catheters have been passed into dilated abdominal veins and portal blood thus obtained. Portal venograms have been obtained by similar methods. These methods have evident limitations and preclude a more general investigation of the portal blood circulation in man. It occurred to one of us (H.L.S.) that access to the portal vein through the intact abdominal wall should be possible, and the senior author (H.R.B.) conceived the idea of an approach through the liver. He subsequently developed the percutaneous, trans-hepatic technic of portal venipuncture described below with the assistance of the two junior authors (L.P.W. and K.H.K.).

The portal vein is 7 to 8 cm in length, extending from the level of L2 to the porta hepatis at the junction of L1 and T12(1). The portal vein divides to the right and left just before it enters the liver(2), the right branch being shorter and thicker than the left. The division of the portal vein usually takes place at the level of T12, in the transverse plane at approximately 15 degrees caudal to the right in the frontal plane(3). In a transverse cross-section(2) which passes through the twelfth thoracic vertebra the larger radicles of the portal vein within the liver substance lie to the right of the midline so that lines to midpoint of the anterior surface make angles of 10 to 45 degrees with the medial plane. At this level the liver abuts directly upon the anterior abdominal wall so that no other organ is interposed. On the basis of these anatomical considerations, a needle was successfully inserted into the hepatic portal vein of a cadaver. This was followed by studies on living patients in some of which contrast media was injected and venograms were obtained which demonstrated the intrahepatic portion of the hepatic portal vein.

Method. The patient is placed in the semi-recumbent position inclined upwards approximately 10 degrees. The epigastrium is prepared aseptically and a midline point 1 cm below the xiphoid process is anesthetized with procaine, including the deeper skin, subcutaneous tissues and parietal peritoneum. No additional analgesia is achieved by infiltration of the visceral peritoneum of the liver and of the liver parenchyma. A 16- to 20-gauge needle, 20 cm long, is inserted through the skin and subcutaneous tissue at an angle of 20 degrees to the right. If the liver is of average size, the needle is inserted in the transverse plane without upward or downward deflection. If the liver is enlarged, the needle is best inclined 5 to 15 degrees caudally in addition to the lateral angulation. The needle is advanced steadily and slowly in the proper direction until a sensation of firm resistance is encountered, indicating entrance into the liver. The needle is then advanced slowly until blood can be aspirated or the 15 cm mark is reached. Continual aspiration is attempted as the needle is being advanced. When blood is obtained, a hemostat is clamped firmly about the needle at the skin surface to secure its position. Respiratory movements will move the hub of the needle, but they are minimized in the semi-recumbent position. Nevertheless, the patient should be cautioned not to breathe deeply. Portal blood has shown an increased tendency to clot. Portal blood can be identified by its color, since it is neither as red as arterial blood nor as dark as peripheral venous blood. If the needle is aimed 5 to 10 degrees cranially and 10 to 15 degrees to the right, the larger radicles of the hepatic vein may be entered. Blood from the hepatic vein contains less oxygen than that from the portal vein and closely resembles peripheral venous blood.

Samples for glucose and oxygen determinations have been obtained from the portal vein, a peripheral vein, and an artery simultaneously or within 2 minutes of each other. In most instances the patient had been given sugar by mouth ½ to one hour before the sampling, which resulted in a greater elevation of glucose content of the portal blood than that obtained from peripheral sites. This served to confirm that the blood was obtained from the portal system. Fourteen portal venipunctures have been successfully accomplished on 12 patients without immediate or late reaction. The procedure was first attempted on cadavers, then on patients with a short life expectancy because of cancer. Following their demise, examination of the tract of the needle in the liver revealed that it could be followed into a portal radicle without evidence of bleeding or gross trauma.

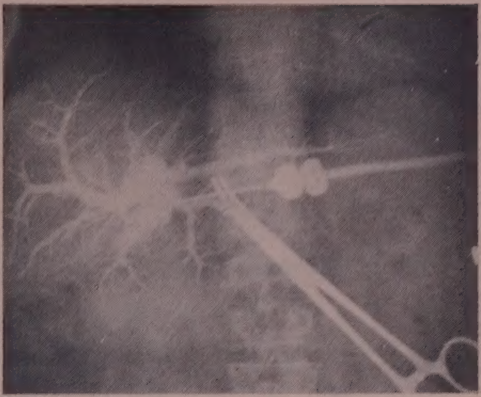


FIG. 1. Portal venogram by direct venipuncture.

In 3 patients who exhibited a hemorrhagic diathesis, a dilute thrombin solution was injected along the tract of the needle after it had been removed from the vein and as it was being withdrawn from the surface of the liver. In 7 others with bleeding tendencies thrombin was not employed and there were no untoward reactions. Following the procedure no special precautions have proved necessary and frequently the patients have walked back to the ward.

In some of the patients who had a portal venipuncture, venograms were performed and were of as good quality as many that had been obtained by injection of a contrast medium during laparotomy (Fig. 1). This will be reported later(4).

Conclusion. This technic permits relatively easy access to the portal venous system in man. It should afford many opportunities for clinical investigations, including portal venography.

---

1. Eycleshymer and Schoemaker, *A cross-section anatomy*. Appleton Century 1950, New York.

2. Gilfillan, R. S., *Arch. Surg.*, 1950, v61, 449.

3. Morris, Henry, *Human anatomy*. Blakiston 1944, Philadelphia.

4. Steinbach, H. L., Bierman, H. R., and Wass, W. A., to be published.

Received March 3, 1952. P.S.E.B.M., 1952, v79.

Tissue Electrolytes in Alloxan-Diabetic Rats with Ketoacidosis.* (19443)

Harvey C. Knowles, Jr.,† and George M. Guest.

From the Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati.

Metabolic balance studies on diabetic human subjects and experimental animals have afforded abundant evidence that the development of ketonemic acidosis is attended with large urinary losses of water, electrolytes and nitrogen. The nature of the losses suggests that increased cellular catabolism in acidosis leads to a considerable depletion of intracellular materials without breakdown of whole tissues. It may be surmised that functional derangements which occur during severe and prolonged acidosis depend less on changes in constituents of extracellular fluids, much studied and well known, than on changes of intracellular constituents, which have been relatively little studied. The investigations reported here were undertaken to amplify existing knowledge by direct analysis of tissues.

Following is a preliminary report of changes in liver and skeletal muscle of alloxan-diabetic rats in severe ketonemic acidosis. More extensive studies on other animals and other tissues will be reported later.

Methods. Adult white rats from Rockland Farm, weighing around 300 g, fed Purina Chow diet, were kept in glass metabolism cages for collecting urine(1). Three groups, of 36 to 40 rats in each, were studied: a control group, not treated with alloxan, and two alloxan-treated groups. In the latter, diabetes was induced by intravenous injections of alloxan, 50 mg/kg, after a 24- to 30-hour fast. One group was allowed food 24 hours after the injection of alloxan, then starved to induce ketoacidosis. The second alloxan-treated group was fed and treated with NPH insulin, 3 to 5 units per day, until the animals recovered their original weight, usually a period of 7 to 10 days; then ketoacidosis was induced by withholding food and insulin. In both groups severe ketoacidosis developed as a rule within 3 to 4 days, as described in a previous paper from this laboratory(2). All were anesthetized by intraperitoneal injection of nembutal for the taking of blood (drawn from the inferior vena cava) and tissue samples. The whole liver was removed, the gall bladder dissected away, and excess blood removed by blotting with filter paper. The thigh muscles were stripped, dissected free from gross fat and connective tissue. The tissues were transferred as quickly as possible to weighing bottles. Tissue water was determined by weighing and drying the samples at 105°C to constant weight. The dried tissues, liver and thigh muscles, were ground to obtain a homogenous powder. Fat content was determined by extracting the powder with petroleum and ethyl ether in a Soxhlet reflux apparatus. Weighed samples of the fat-free powder were used for determinations of Cl by the method of Van Slyke(3), and of nitrogen by the microkjeldahl method; samples were dry ashed for determinations of sodium and potassium with the Weichselbaum-Varney flame photometer. Samples of blood

---

* Aided by a grant from the Life Insurance Medical Research Fund.

† Postdoctorate Research Fellow, National Institutes of Health, Bethesda, Md.

TABLE I. Data on Blood Serum, Muscle, and Liver of Normal Rats and Diabetic Rats with Ketoacidosis. Mean of 12 determinations with standard deviations in each group.

Groups	Sugar, mg/100 cc	Ketones,* mg/100 cc	pH	CO_2, meq/l
		Serum		
Normal	121†		7.45	28.4
	±15		±.04	±2.2
Acidotic 1	686	174	7.08	9.5
	±126	±73	±.10	±2.9
Acidotic 2	746	211	6.99	8.8
	±199	±98	±.06	±2.0

Muscle

Groups	Fat % of total solids	Per 100 g fat free solids				
		H_2O, g	N, g	Cl, meq	Na, meq	K, meq
Normal	7.0	308.4	14.77	5.10	8.22	45.0
	±1.1	± 4.4	±.56	± .57	± .65	±1.84
Acidotic 1	10.0	272.8	15.16	5.25	8.26	38.23
	±2.4	±15.0	±.25	±1.06	±1.43	±2.44
Acidotic 2	5.5	271.4	15.34	5.23	8.87	39.69
	±1.5	± 5.9	±.68	±1.43	±1.01	±2.51

Liver

Groups	Fat % of total solids	Per 100 g fat free solids				
		H_2O, g	N, g	Cl, meq	Na, meq	K, meq
Normal	7.34	265.3	11.94	10.34	8.78	32.9
	±1.8	±27.9	±.74	±2.91	±1.24	±3.25
Acidotic 1	38.9	330.4	13.62	11.71	11.41	32.0
	±16	±33.4	±.37	±3.82	±1.73	±4.55
Acidotic 2	20.7	310.8	13.50	11.18	11.26	34.4
	±10.9	±21.7	±.36	±1.57	±1.47	±2.62

Acidotic Group 1: Rats inj. with Alloxan, fed one day, then allowed to develop ketoacidosis immediately, receiving no insulin or food.

Acidotic Group 2: Rats inj. with Alloxan, fed and treated with insulin 7 days; acidosis induced by withholding insulin and food.

* Ketones expressed as acetone.

† "Normal" blood sugar values determined 1 hr after feeding.

were delivered from a syringe under oil for determinations of serum pH(4) and CO_2 content(5). Sugar in whole blood or serum was determined by the method of Nelson(6). Ketones in diluted plasma were estimated by a micromethod(7) employing the acetone test tablets or powder commonly used for qualitative tests for acetone in urine, with appropriate color standards.

Results. Data from analyses of blood, skeletal muscle and livers of the control group and two acidotic groups of rats are listed in Table I. High values for serum sugar and ketones, and low values for serum pH and CO_2 content were approximately the same in the two acidotic groups. Muscle of both acidotic groups showed a lowered content of water and of potassium, while the chloride and sodium values were not significantly different from those of the controls; the slightly higher value for nitrogen content is probably significant (p between 0.05 and 0.02). In liver the fat content was greatly increased, higher in group 1 than in group 2; the content of water, sodium and nitrogen was significantly increased, but the potassium and chloride values were not changed, compared with the controls.

Discussion. The high fat content of muscle in the acidotic group 1 may be ascribed to immediate cellular toxic effects of the alloxan apart from its diabetogenic action. In relation to fat-free solids the potassium concentration decreased 15 and 12% respectively in the two groups. It is of interest to note here that a number of investigators(8-10) studying the effects of prolonged low-potassium diets, designed to produce potassium deficiency, have found increases in the sodium content of muscle when the potassium content decreased. The failure in the present experiments of sodium to increase with lowered potassium may be attributed to the dehydration and acidosis and to the short duration of these experiments.

The high fat content of the livers of the acidotic rats is presumably associated with the development of severe ketosis and with toxic effects of alloxan, which is known to produce some hepatic necrosis. Although the degree of ketonemia and acidosis seemed alike in the two groups, the fat content of the livers was less in the second group when ketosis was induced after 7 days of feeding and insulin control, suggesting some recovery from early toxic effects of the alloxan. Increased contents of water and sodium may be considered to be interdependent. The lack of change of potassium in the livers of the acidotic animals conforms with observations by other investigators mentioned above that potassium deficiency (induced by diet) was not attended with any change in liver potassium.

The finding in the acidotic rats of a higher than normal nitrogen content in liver and muscle, relative to the total solids, is in accord with the concept that acidosis leads to a greater loss of labile non-protein constituents than of protein from body tissues.

Summary. Data are reported from analyses of tissues of normal rats and of alloxan-diabetic rats with severe ketoacidosis induced by withholding food and insulin 3 to 4 days. In muscle of the acidotic rats, compared with the normal controls, the concentration of water and of potassium decreased, and the concentration of nitrogen increased slightly, with values for sodium and chloride showing no significant change. In liver there was a gain of water, sodium and nitrogen, with no change in concentration of chloride or potassium. Livers of the acidotic rats showed a greatly increased fat content.

---

1. Guest, G. M., Brodsky, W. A., and Nelson, N., *Metabolism*, 1952, v1, 89.
2. Brodsky, W. A., Nelson, N., and Guest, G. M., *Metabolism*, 1952, v1, 68.
3. Van Slyke, D. D., *J. Biol. Chem.*, 1923, v58, 523.
4. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, 1924, v61, 695.
5. Van Slyke, D. D. and Neil, J. M., *J. Biol. Chem.*, 1924, v61, 523.
6. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.
7. Guest, G. M., and Lestradet, H., to be published.
8. Heppel, L. A., *Am. J. Physiol.*, 1939, v127, 385.
9. Orent-Keiles, E., and McCollum, E. V., *J. Biol. Chem.*, 1941, v140, 337.
10. Gardner, L. I., Talbot, N. B., Cook, C. D., Berman, H., and Uribe, C., *J. Lab. and Clin. Med.*, 1950, v35, 592.

Received March 6, 1952. P.S.E.B.M., 1952, v79.

ERRATUM

Article 19259, Vol 79, p. 21, title should read "Ineffectiveness of Parenteral Pyridoxine . . ."